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α-Phenyl-*N*-*tert*-butyl nitrone (PBN) derivatives: Synthesis and protective action against microvascular damages induced by ischemia/reperfusion

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Abstract—Nitrones **4**–**7**, structurally related to PBN (**1**), were prepared by reaction of the corresponding aromatic aldehydes with *N*-*tert*-butyl hydroxylamine. The protective effects of these nitrones against microvascular damages in ischemia/reperfusion in the 'hamster cheek pouch' assay were studied and **1**, as well as **4a**, **4b**, and **7** (derived from piperonal, *O*-benzyl vanillin, and furfural, respectively), showed to be more active than shark cartilage or α-tocopherol. No correlation was found between the protective effect of these nitrones and their log*P* (partition coefficient) or their capacity to trap $^{\cdot}OH$ and $^{\cdot}CH_3$ radicals.

1. Introduction

Nitrones were first recognized as spin traps in late sixties[1] and since then have been extensively used for the detection and identification of transient radical species in chemistry.[2] In biological systems, they have been used for in vivo identification of free radicals formed after the administration of drugs and xenobiotics.[2,3] Rate constants for addition of radicals to nitrones are extremely fast and were found to be independent of the structure of the nitrone. Of more relevance is the persistence of the spin adduct and the uniqueness of the EPR spectrum.[4] PBN (**1**), one of the most studied nitrones, forms stable spin adducts by reaction with carbon-centered radicals but is less useful for detecting oxygen-centered radicals, due to the instability of the corresponding spin adducts.[5a] However, 4-hydroxy PBN is formed by attack of $^{\cdot}OH$ radicals at the phenyl ring in **1** and the identification of this metabolite has been used as evidence for the occurrence of these radicals.[5] The lifetime of spin adducts formed by reaction of $^{\cdot}OH$ radicals with **1** and derivatives was studied.[4,6] In addition to detecting the presence of radicals and identifying their structures, nitrones have also been used to prevent or reduce the damage caused by radicals in biological systems. It was reported that PBN (**1**, Fig. 1) inhibits the oxidation of lipoproteins,[7] prevents liver injury originated from administration of a mixture of CCl_4 and ethanol to rats,[8] and reduces oxidative damage to erythrocytes and peroxidation of lipids caused by phenylhydrazine.[9] Since the early studies of Novelli and co-workers, the neuroprotective activity of **1** has been extensively investigated.[10] Due to the good blood–brain barrier penetration, **1** protected gerbils from lethality induced by global brain stroke[11] and protected mice against MPTP toxicity (a Parkinson's disease model).[11] PBN (**1**) also has been shown to extend life span in mouse and rat models.[10]

Keywords: PBN derivatives; Protective action against microvascular damages; Ischemia/reperfusion.
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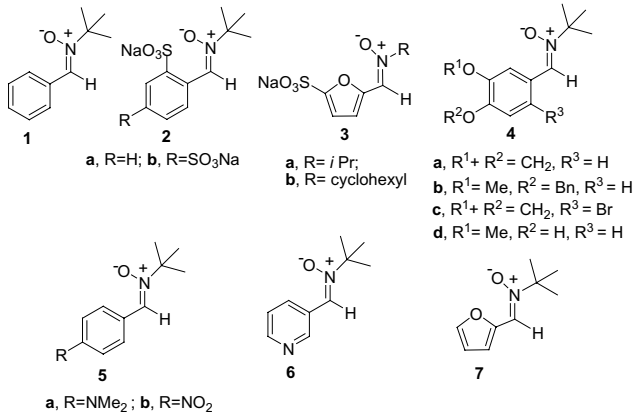


Figure 1. PBN (**1**) and PBN-like nitrones **3**–**7**.

In an effort to optimize the biological profile of **1**, a wide structural diversity of PBN-like nitrones has been designed and synthesized. In rats, post-ischemic administration of nitrone **2a** (S-PBN) provided substantial neuroprotective properties in ischemia/reperfusion nerve injury.[12a] Nitrone **2b** (NXY-059) is an effective neuroprotective agent in rat models of transient and permanent focal ischemia. This compound improved neurological function and reduced infarct volume in a primate model of permanent focal ischemia, even when given 4 h post-occlusion, and is now under clinical trials (phase 3).[12b] Furylnitrones such as **3a**,**b** showed low toxicity and reduced infarct volume in an in vivo stroke model in rodents.[13] Nitrone **4a** was patented for treatment of neurodegenerative, autoimmune, and inflammatory diseases, and as analytical reagent for detection of free radicals.[14] New PBN derivatives have been synthesized in order to optimize the biodisponibility[12,15] and to reduce the hypothermia, a side effect associated with **1**.[16] The inhibition of early phase carcinogenesis in the liver of rats[16] and the protection against myelotoxicity and adriamycin-induced cardiotoxicity,[17] were also reported for nitrones.

The mechanism of action of **1** is not fully understood. The protective effect against MPTP toxicity in mice was initially attributed to an anti-oxidant activity, but recent results show that it acts only as moderate retarder of oxygen uptake at relatively high concentrations.[18] This action is independent of hydroxyl radical trapping and seems to be associated with carbon-centered radical trapping. It was also shown that **1** acts on secondary mitochondrial focal ischemia. It appears that the neuroprotective activity of nitrones involves the inhibition of enhanced signal transduction that mediates the upregulation of genes, which produce neurotoxic products.[10b] It is not known exactly why the nitrones possess anti-aging activity but they have been shown to quell increased signal transduction processes associated with enhanced pro-inflammatory cytokine mediated events which can interfere in some unknown steps preventing receptor triggered MAP kinase phosphorylation cascades.[10c]

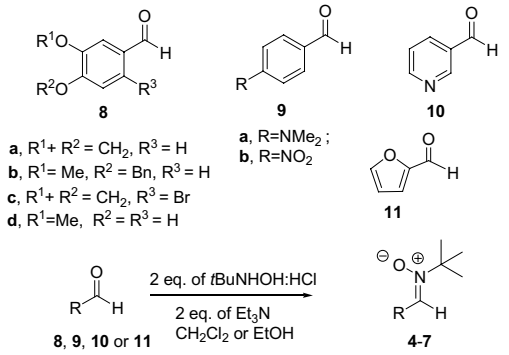
Previous reports suggest that PBN and/or PBN spin adducts undergo decomposition to form *tert*-butyl hydroxylamine and/or the corresponding hydronitroxide, which can subsequently be oxidized to nitroso-*tert*-butane (*t*-NB). The generation of this radical may play a role on cardiovascular and central nervous systems effects observed for **1**, but this matter is rather controversial.[19a] It was also shown that nitrones exert a potent vasodilatatory effect in isolated perfused rat heart.[19b] Imidazoline nitrones under in vitro conditions trapped $^{\cdot}NO$ release, leading to adducts detected by EPR. These compounds inhibited SPN-induced vasodilation.[19b]

In this paper, we describe the synthesis and the protective effect of nitrones **1** and **4**–**7** against microvascular damages induced by ischemia/reperfusion in the 'hamster cheek pouch' assay.[20] The results were compared with their capacity to trap $^{\cdot}OH$ and $^{\cdot}CH_3$ radicals and their partition coefficient (log *P*). Nitrones **4b**–**d** were prepared for the first time for this work. Nitrone **7**[21] was previously reported in a study to determine the configuration of the double bond in a series of aldonitrones. Spin trapping properties of **1**, **5a**,**b**, and **6** have been extensively studied.[5,6,22]

2. Results

2.1. Chemistry

Several methods are described in the literature to prepare nitrones[6] and we chose the protocol described by Dandoni et al.,[23] used to synthesize *N*-benzyl nitrones through the reaction of aldehydes with *N*-benzyl hydroxylamine, chlorohydrate under very mild conditions (Et_3N, $CHCl_3$, $MgSO_4$, rt, ~4 h). In contrast with these results, when the bulky *N*-*tert*-butyl hydroxylamine was used as nucleophile the reactions were much slower (Scheme 1 and Table 1). The transformation of more reactive aldehydes **8c**, **9b**, **10**, **11** into nitrones **4c**, **5b**, **6**, and **7** could be accomplished at rt, but good yields were obtained only in the presence of 2 equiv of *N*-*tert*-butyl hydroxylamine, after several days (entries 3, 6, 7, and 8). Aldehydes **8a**,**b** were less reactive and the formation of **4a**,**b** required reflux in CH_2Cl_2 (entries 1 and 2). Aldehydes **8d** and **9a** were yet less reactive and the formation of the corresponding nitrones, **4d** and **5a**, required the use of ethanol, at reflux (entries 4 and 5).



Scheme 1. Synthesis of nitrones **4**–**7** from aldehydes **8**–**11**.

Table 1. Synthesis of nitrones **4**–**7**, conditions and yields

Entry	Aldehyde	Nitrone	Condition	Time (days)	Yields (%)
1	**8a**	**4a**	**b**	3	51
2	**8b**	**4b**	**b**	3	66
3	**8c**	**4c**	**a**	10	63
4	**8d**	**4d**	**c**	2	45
5	**9a**	**5a**	**c**	3	36
6	**9b**	**5b**	**a**	7	96
7	**10**	**6**	**a**	4	81
8	**11**	**7**	**a**	7	80

Compound **a**, CH_2Cl_2, $MgSO_4$ or Na_2SO_4, rt; **b**, CH_2Cl_2, $MgSO_4$ or Na_2SO_4, reflux; **c**, EtOH, $MgSO_4$ or Na_2SO_4, reflux.

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 **4c** and **4d** on the basis of NOESY NMR spectra in CD_3Cl and comparison with spectroscopic data available in the literature.[24]

2.2. Pharmacology

Tissue ischemia occurs when inadequate blood supply takes place and it is now clear that reperfusion to an ischemic area initiates a complex cascade of pathological events that could potentially lead to cellular dysfunction and necrosis. Reactive oxygen species formed during reperfusion promote a pro-inflammatory stimuli, modify the expression of adhesion molecules on the surface of leucocytes and endothelial cells, and reduce the levels of nitric oxide in the muscle, a potent anti-adhesive substance.[20] This latter effect is exacerbated by a post-ischemic decline in tissue levels of NO synthase. As a consequence, the leucocytes start 'rolling' closer to the endothelial surface, which is followed by adherence to endothelial cells of post-capillary venules. Activated leucocytes migrate into the tissue, inducing microvascular barrier dysfunction via release of oxidants and hydrolytic enzymes.[20] Thus, enhanced vascular macromolecular leakage is one of the earliest signs of microvascular dysfunction elicited by ischemia/reperfusion. The increased permeability leads to an enhanced fluid filtration, with a consequent increase in interstitial fluid pressure, which could physically compress the capillary and lead to the development of the 'no-reflow' phenomenon. Several different experimental models have been devised to study the effect of ischemia and reperfusion in vivo but not many models offer the ability to observe acute changes at the microcirculatory level. The thin distal part of the hamster cheek pouch is highly vascularized and well suited for intravital microscopy studies of the post-ischemic events in the microcirculation and was used in this work.[20]

With nitrones **1** and **4**–**7** in hands, their protective effect against microvascular damages during ischemia/reperfusion in the 'hamster cheek pouch' assay was investigated (Table 2).

Under control conditions, the number of leakage sites observed during reperfusion in the cheek pouch after 30 min of total ischemia was 123.3 ± 4.7 per cm^2 (mean ± SD). After a single oral dose of 3 mg/kg body weight of **1** and derivatives **4**–**7**, the inhibition of macromolecular permeability increase induced by ischemia was measured. Nitrones **4a**,**b** in which the aromatic ring is substituted at positions 3 and 4 by alkoxy groups, were almost as potent as **1** (entries 1, 2, and 3). The presence of the bromine atom at the *ortho*-position of the nitrone group, as in **4c**, decreased the potency ~3 times (entry 4) and compound **4d** (entry 5), bearing a phenol group at the 4-position, was also less potent than **1** (~2 times). Nitrogen containing nitrones **5a**, **5b**, and **6** were ~2 time less potent than **1**. Finally, the furan-derivative **7** (entry 9) was almost equipotent to **1**.

Table 2. Protective effect of nitrones **1** and **4**–**7** against ischemia/reperfusion injury in the 'hamster cheek pouch' assay

Entry	Compound	Number of leakage sites/cm^2	
		10 min	15 min
1	**1**	38	35
2	**4a**	44	38
3	**4b**	40	35
4	**4c**	118	109
5	**4d**	108	85
6	**5a**	75	55
7	**5b**	90	78
8	**6**	68	60
9	**7**	49	33
10	α-Tocopherol[a]	50	45
11	Shark cartilage[b]	46	40

Number of leaks in the absence of compound (control) = 125 leaks/cm^2 at 10 min.
[a] 10 mg/kg.
[b] 100 mg/kg.

2.3. EPR and log *P*

In order to know if the protective effects of nitrones **1** and **4**–**7** could be ascribed to their activity as radical trapping agents, their capacity to trap $^{\cdot}CH_3$ and $^{\cdot}OH$ radicals was evaluated in a first approximation by electronic paramagnetic resonance (EPR) (Figs. 2 and 3), by comparing the EPR peak intensities after three minutes of incubation. The data in Figures 2 and 3 show that nitrones **4**–**7** are more efficient to trap $^{\cdot}CH_3$ than

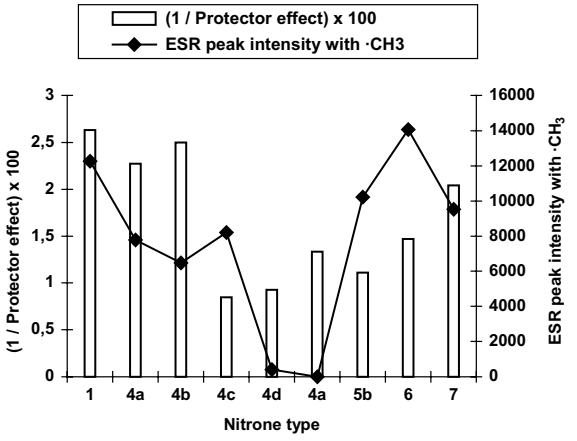


Figure 2. Protector effect of nitrones **1** and **4**–**7** versus ESR peak intensity with the $^{\cdot}CH_3$ radical.

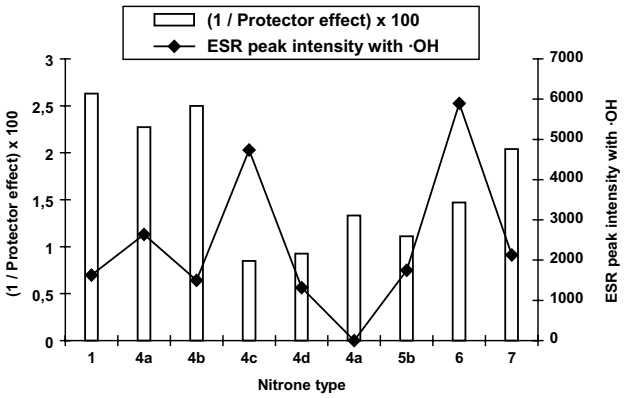


Figure 3. Protector effect of nitrones **1** and **4**–**7** versus ESR peak intensity with the ·OH radical.

·OH radicals, as previously observed for PBN (**1**) and some derivatives.[5] As shown by the peak intensity in the EPR spectra, nitrone **6** was more efficient than **1** to trap both $^{\cdot}CH_3$ and ·OH radicals. Nitrones **4a**, **4b**, and **7** were less efficient than **1** to trap $^{\cdot}CH_3$, while their affinities for ·OH were similar. Nitrones **4c** and **6** were the more efficient to trap ·OH radicals. Finally **4d**, bearing a free phenol group in the structure, presented very low efficiency to trap both radicals, while **5a**, substituted by a free amino group, did not trap these radicals at all. PBN (**1**), the most active compound, was the second more efficient to trap $^{\cdot}CH_3$, while **6**, the most efficient to trap $^{\cdot}CH_3$, was only moderately active (Fig. 2). Compounds **4a**,**b**, and **7** were almost as active as **1**, trapped $^{\cdot}CH_3$. Even compounds **4d** and **5a**, which did not trap $^{\cdot}CH_3$, presented some protective effect. As also observed for neuroprotective[10a] and anti-aging actions,[10b] it is clear from these data that the capacity of trapping $^{\cdot}CH_3$ does not appear to be the only factor governing the protective effect. No correlation could also be found between the protective effect and the capacity to trap ·OH (Fig. 3). It seems that the nature of the aromatic ring (carbocycle or heterocycle) and the presence of substituents at the benzene ring in nitrones **4**–**7** provide different sites for interaction with the biophase, modulating the protector effect through non-identified mechanisms of action.

Nitrones **4**–**7** presented different partition coefficients ($\log P$). Except for **4a** and **6**, which are less soluble in lipids than **1**, other derivatives are as soluble as or more soluble than **1**. As shown in Table 3, no correlation could be found between the protective effect and $\log P$ (Table 3). The theoretical $\log P$ was calculated using VCCLAB software and experimental $\log P$ was determined by reversed-phase liquid chromatography method. The experimental and calculated values for PBN (**1**) and *para*-nitro-PBN (**5b**) are in agreement with the octanol–water partition coefficients previously reported,[25] 1.2 and 1.4, respectively.

Table 3. Comparison between $\log P$ values and biological activities

Nitrones	$\log P$ (HPLC)	$\log P$ (VCCLAB® software)	Microvascular protection (10 min)
1	1.50	1.40	38
4a	0.57	0.76	44
4b	2.68	2.56	40
4c	1.69	1.52	118
4d	1.58	1.46	108
5a	1.73	1.74	75
5b	1.39	1.50	90
6	0.21	0.31	68
7	1.10	1.05	49

3. Conclusions

Nitrones **1**, **4a**, **4b**, and **7** can be considered as very active against microvascular damages induced by ischemia/reperfusion in the 'hamster cheek pouch' preparation. They were more active than shark cartilage or α-tocopherol, given orally for 10 days in a higher dose[26], and are candidates for further evaluations. Moreover, diseases such as hypertension and diabetes have an increased production of oxygen species and such compounds could be considered as candidate in the therapy of these diseases.

4. Materials and methods

$Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$, Na_2SO_4, silica, silica gel 60 F-254 plates, DMF, DMSO, Eppendorfs, and borosilicate glass micropipettes for EPR measurements were purchased from VWR International (Strasbourg, France). Other chemicals were purchased from Sigma–Aldrich–Fluka Co. Column chromatography was carried out using 200–400 mesh chromagel. Melting points were determined on 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 (δ) are reported in ppm relative to tetramethylsilane (0 ppm) and the following multiplicity abbreviations were used: s, singlet; d, doublet; t, triplet; q, quadruplet; 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. EPR spectra were obtained at X-band at room temperature on a Bruker EMX-8/2.7 (9.86 GHz) equipped with a high-sensitivity cavity (4119/HS 0205) (Bruker Wissembourg, France).

4.1. Synthesis of α-aryl-*tert*-butyl nitrones (4a–c, 5b, 6, and 7)

To a mixture of aromatic aldehyde (5 mmol), triethylamine (11.0 mmol; 1532 μL), and dry Na_2SO_4 (2 g) in CH_2Cl_2 (40 mL) was added *N*-*tert*-butyl hydroxylamine hydrochloride (10 mmol; 1256 mg). The mixture was stirred at room temperature or at 60 °C (for **3a**,**b**) for 4–10 days. After this time, the reaction mixture was filtered, the solid layer was washed with CH_2Cl_2, and the combined organic layer was evaporated under reduced

pressure. The crude product was purified by column chromatography (solvent: cyclohexan/ethyl acetate, 60:40).

Compound **4a**. Mp 87 °C, R_f 0.35 (CH_2Cl_2/ethanol, 98:2). UV (CH_3CN) λ_{max} nm (ε): 318 (70980). IR (KBr) cm^{-1}: 3089, 2981, 2897, 1597, 1571, 1503, 1482, 1442, 1365, 1296, 1195, 1119, 1036, 931, 889, 832. ^1H NMR ($CDCl_3$, 300 MHz): δ 8.22 (s, 1H), δ 7.56 (d, J = 8.1 Hz, 1H), δ 7.46 (d, J = 2.4 Hz, 1H), δ 6.87 (d, J = 8.1 Hz, 1H), δ 6.01 (s, 2H), δ 1.61 (s, 9H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 147.0 (2(*C*–$COCH_2$)), δ 129.7 (*C*H–NO), δ 125.0 (*C*–CNO), δ 124.0 (*C*–CCH), δ 108.6 (*C*H–CHC), δ 108.2 (*C*H–C–CCH), δ 101.0 (*C*OO), δ 70.3 ($C(CH_3)_3$), δ 28.2 (3CH_3).

Compound **4b**. Mp 104 °C, R_f 0.18 (CH_2Cl_2/ethanol, 98:2). UV (CH_3CN) λ_{max} nm (ε): 316 (16258). IR (KBr) cm^{-1}: 3098, 3003 2955, 2868, 1585, 1504, 1454, 1420, 1361, 1272, 1198, 1167, 1148, 1015, 883, 816, 769. ^1H NMR ($CDCl_3$, 300 MHz): δ 8.56 (s, 1H), δ 7.28–7.46 (m, 7H), δ 6.89 (d, J = 8.7 Hz, 1H), δ 5.21 (s, 2H), δ 3.98 (s, 3H), δ 1.61 (s, 9H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 149.4 (*C*–OCH_2), δ 149.0 (*C*–OCH_3), δ 136.6 (*C*–CH_2O), δ 129.7 (*CH*–NO), δ 128.5 (*CH*–CHC), δ 127.9 (*CH*–CHCH), δ 127.2 (CH–*CH*–C), δ 124.8 (*C*–CHNO), δ 123.0 (*CH*–CCHNO), δ 113.0 (*CH*–CO), δ 111.4 (*CH*–$COCH_3$), δ 70.7 (CH_2–C_6H_5), δ 70.2 (*C*–$(CH_3)_3$), δ 55.9 (CH_3–O), δ 28.3 (3CH_3). HRMS (Electrospray): *m*/*z* calcd for $C_{19}H_{23}NO_3$, 313.3910. Found: 313.1741.

Compound **4c**. Mp 121 °C, R_f 0.50 (cyclohexane/ethyl acetate, 65:35). UV (CH_3CN) λ_{max} nm (ε): 232 (34518). IR (KBr) cm^{-1}: 3105, 2971, 2894, 1615, 1555, 1507, 1468, 1415, 1359, 1267, 1194, 1140, 1034, 921, 879, 835, 797. ^1H NMR ($CDCl_3$, 300 MHz): δ 9.03 (s, 1H), δ 7.99 (s, 1H), δ 7.08 (s, 1H), δ 6.03 (s, 2H), δ 1.62 (s, 9H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 149.0 (*C*–Br), δ 147.0 (*C*–CBr), δ 128.8 (*C*H–NO), δ 123.8 (*C*–CO), δ 116.3 (*C*–CO), δ 112.9 (*C*H–CBr), δ 109.0 (*C*H–C–CBr), δ 102.0 (*C*OO), δ 71.5 ($C(CH_3)_3$), δ 28.2 (3CH_3). HRMS (Electrospray): *m*/*z* calcd for $C_{12}H_{14}NO_3Br$, 300.1490. Found: 300.0218.

Compound **7**. Mp 68.5 °C, R_f 0.38 (cyclohexane/ethyl acetate, 50:50). UV (CH_3CN) λ_{max} nm (ε): 303 (19184). IR (KBr) cm^{-1}: 3102, 2981, 2936, 1575, 1551, 1481, 1399, 1357, 1249, 1213, 1117, 1007, 895, 826, 766, 748. ^1H NMR ($CDCl_3$, 300 MHz): δ 7.77 (td, J = 3.4 Hz, 1H), δ 7.72 (s, 1H), δ 7.48 (dd, J = 1.7 Hz, 1H), δ 6.60 (dd, J = 2.0 Hz, 1H), δ 1.58 (s, 9H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 147.6 (CH–*C*–O), δ 143.4 (*CH*–NO), δ 121.3 (*CH*–O–C), δ 114.7 (*CH*–CH–C), δ 112.2 (*C*H–C), δ 69.6 (*C*–$(CH_3)_3$), δ 28.0 (3CH_3).

4.2. Synthesis of α-aryl-*tert*-butyl nitrones (4d and 5a)

To a mixture of aromatic aldehyde (5 mmol), triethylamine (11.0 mmol; 1532 μL), and dry Na_2SO_4 (2 g) in absolute EtOH (10 mL) was added *N*-*tert*-butyl hydroxylamine hydrochloride (10 mmol; 1256 mg). The mixture was stirred at reflux for 2 or 3 days. After this time, the reaction mixture was filtered, the solid layer was washed with EtOH, and the combined organic layer was evaporated under reduced pressure. The crude product was purified by column chromatography (solvent: cyclohexan/ethyl acetate, 60:40).

Compound **4d** yield 45%, reaction time 2 days. Mp 196 °C. R_f 0.49 (CH_2Cl_2/methanol, 90:10). UV (CH_3CN) λ_{max} nm (ε): 301 (13591). IR (KBr) cm^{-1}: 3007, 2935, 2722, 2590, 1591, 1514, 1462, 1396, 1362, 1301, 1190, 1158, 1095, 1031, 964, 883, 815, 790. ^1H NMR ($CDCl_3$, 300 MHz): δ 8.70 (s, 1H), δ 7.47 (s, 1H), δ 7.20 (d, J = 8.4 Hz, 1H), δ 6.93 (d, J = 8.4 Hz, 1H), δ 6.30 (s, 1H), δ 3.95 (s, 3H), δ 1.62 (s, 9H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 147.6 (*C*–OCH_3), δ 146.0 (*C*–OH), δ 130.0 (*C*H–NO), δ 124.3 (*CH*–CH–COH), δ 123.7 (CH–*C*–CH), δ 114.2 (*C*H–C–OCH_3), δ 110.6 (*C*H–C–COH), δ 70.1 (*C*–$(CH_3)_3$), δ 55.9 (CH_3–O–C), δ 28.3 (3CH_3). HRMS (Electrospray): *m*/*z* calcd for $C_{12}H_{17}NO_3$, 223.2680. Found: 223.1284.

4.3. Spin trapping experiments

$^{\cdot}CH_3$ was generated by Fenton's reagents (Fe^{2+}/H_2O_2, 100 μM/100 μM) in H_2O/DMSO, 40:60 (v/v), 1 mM spin trap. $^{\cdot}OH$ was generated by Fenton's reagents (Fe^{2+}/H_2O_2 100 μM/100 μM) in H_2O/DMF, 60:40 (v/v), 30 mM spin trap.

4.4. HPLC–UV–visible measurement ($\log P_{exp}$)

The system included: an automatic injector (Waters 717 plus), a degasser (Waters In-Line), and a quaternary pump (Waters 600) coupled to a UV–visible detector (Waters 2487). Data acquisition was performed using Waters Millenium software, version 3.2 (Waters, St. Quentin, France). The chromatographic separation was performed on a Supelco INC Lichrosorb, C-18 (5 μm) analytical column (150 × 4.6 mm). The mobile phase [methanol–H_2O (75:25 v/v)] was delivered at a flow rate of 1 mL/min in an isocratic mode and the injected volume was 20 μL. The nitrone and aldehyde solutions were prepared as follows: 1 mg in 1 mL of acetonitrile and then diluted in a final volume of 10 mL.

Determination of log *P* experimental. Nitrones and aldehydes for which log *P* was known were used as reference. Retention times (t_r) and void time (t_0 = 1.55 min for purified water) were determined. The retention factor was calculated ((k'): $k' = (t_r - t_0)/t_r$). The calibration curve was plotted [$\log k'_{references} = f(log\ P_{references})$] and using this curve and $\log k'$ of PBN derivatives, $\log P_{exp}$ were then determined.

4.5. $\log P_{calc}$

$\log P_{calc}$ were calculated with the software from Virtual Computational Chemistry Laboratory (VCCLAB®, http://www.virtuallaboratory.org/lab/alogps/start.html).

4.6. Hamster cheek pouch preparation

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 University of Rio de Janeiro (H36/94). Animals received appropriate laboratory diet (Nuvital, Nuvilab, Paulinia, PR, Brazil). All drugs tested were administered by gavage, at a dose of 3 mg/kg body weight, 30 min before the induction of anesthesia by an intraperitoneal injection of sodium pentobarbital and maintained with α-chloralose administered through the femoral vein. The femoral artery was also cannulated for pressure measurements. Throughout surgery and the subsequent experiment, the temperature of the animals was kept at 37.5 °C with a heating pad controlled by a rectal thermistor. A tracheal tube was inserted to facilitate spontaneous breathing. The cheek pouch was everted gently and pinned with four to five needles into a circular well filled with silicone rubber to provide a flat bottom layer, thus avoiding stretching of the tissue, but preventing shrinkage. In this position, the pouch was submerged in a superfusion solution that continuously flushed the pool of the microscope stage. In order to produce a single-layer preparation, an incision was made in the upper layer so that a triangular flap could be displaced to one side.

Thirty minutes after the completion of the preparative procedure, FITC-dextran 150 (Bioflor HB, Uppsala, Sweden) was given as an intravenous injection of a 5% solution in 0.9% saline. Local ischemia of the cheek pouch was produced by means of a cuff, made of thin latex tubing, which was mounted around the neck of the everted pouch where it leaves the mouth of the hamster. An intracuff pressure of 200–220 mmHg resulted in a complete arrest of microvascular blood flow within few seconds. Throughout the 30-min occlusion period, minor adjustments of blood movements could be seen in the larger vessels.

The fluorescent spots formed at leakage sites could be traced when they reached a certain minimal size and fluorescent intensity. Each was classified as a leakage site when its diameter was >100 μm, expressed by cm^2. The maximum number of leaks occurred 10 min after the onset of reperfusion and it is reported in the result section.

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