

[Revised manuscript]

## **Inhibition of 6-hydroxydopamine-induced oxidative damage by 4,5-dihydro-3H-2-benzazepine *N*-oxides**

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## ABSTRACT

A number of new analogs of 3,3-dimethyl-4,5-dihydro-3*H*-2-benzazepine 2-oxide, structurally related to the nitron spin trap  $\alpha$ -phenyl-*N*-*tert*-butylnitron (PBN), were synthesized and evaluated for their activity in vitro as protectants against oxidative stress induced in rat brain mitochondria by 6-hydroxydopamine (6-OHDA), a neurotoxin producing experimental model of Parkinson's disease (PD). As assessed by a fluorimetric assay, all 2-benzazepine-based nitrones were shown to decrease hydroxyl radicals ( $\cdot$ OH) generated during 6-OHDA autoxidation. The inhibition effects on the  $\cdot$ OH formation shown by the 5-*gem*-dimethyl derivatives, 2-to-4 times higher than those of the corresponding 5-methyl derivatives, were attributed to the flattening effect of the 5-*gem*-dimethyl group on the azepine ring, which should enhance nitron reactivity and/or increase stability of the radical adducts. In contrast, owing to steric hindrance, a methyl group to C-1 diminishes the  $\cdot$ OH-scavenging activity of the nitron group. All the assayed compounds were more potent than PBN as inhibitors of 6-OHDA-induced lipid peroxidation (LPO) and protein carbonylation (PCO), taken as an indicator of mitochondrial protein oxidative damage. The most promising antioxidant (compound **11**), bearing 5-*gem*-dimethyl and spiro C-3 cyclohexyl groups, highlighted in this study as the best features, inhibited LPO and PCO with IC<sub>50</sub> values of 20 and 48  $\mu$ M, respectively, showing a potency improvement over PBN of two order magnitude. Both LPO and PCO inhibition potency data were found primarily related to the  $\cdot$ OH scavenging activities, whereas lipophilicity plays a role in improving the LPO (but not PCO) inhibition, as a statistically valuable two-parameter equation proved.

*Keywords:* 2-Benzazepine nitrones – Neuroprotection - Radical scavengers - 6-Hydroxydopamine - Lipid peroxidation - Protein carbonylation

*Abbreviations:* PD, Parkinson's disease; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; 6-OHDA, 6-hydroxydopamine; MPTP, *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PBN,  $\alpha$ -phenyl-*N*-*tert*-butylnitron; ROS, reactive oxygen species;  $\cdot$ OH, hydroxyl radical; LPO, lipid peroxidation; RCS, reactive carbonyl species; PCO, protein carbonylation; THA, terephthalic acid; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; MDA, malondialdehyde; MAO, monoamine oxidase; BSA, bovine serum albumin; SDS, sodium dodecylsulfate.

## 1. Introduction

Free radical-induced oxidation of cell macromolecules (lipids, proteins, DNA, etc.) is implicated in several human pathogenic processes, including cardiovascular diseases (e.g., stroke, atherosclerosis) [1] and inflammatory (e.g., rheumatoid arthritis) [2]. Over the last two decades, significant accumulating evidence has also shown that generation of reactive oxygen species (ROS) and oxidative damage in the central nervous system (CNS) are major events occurring in Parkinson's disease (PD) [3] and other neurodegenerative disorders, such as Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS), as well as in aging processes [4]. Despite a large body of biochemical data gathered from human brain autopsy studies, the cause of neurodegeneration in PD has not yet been completely established. Nevertheless, animal models, based on the toxic damage induced by the neurotoxins 6-hydroxydopamine (6-OHDA) [5], *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [6] and related compounds [7,8], have shown that iron-dependent oxidative stress, increased levels of iron and monoamine oxidase (MAO)-B activity and depletion of antioxidants (e.g., glutathione, GSH) play major roles in PD. Indeed, antioxidants, monoamine oxidase (MAO)-B inhibitors and iron chelators, have shown neuroprotective effects in animal models either in vitro and in vivo [9-13], but unfortunately they failed in the PD human therapy [3] when administered as single drugs.

6-OHDA, used in this and previous studies [14] for inducing oxidative stress associated with the PD-related dopaminergic neuron loss, is readily autoxidized and oxidatively deaminated by MAO, yielding hydrogen peroxide ( $H_2O_2$ ) and the corresponding *p*-quinone.  $H_2O_2$  may generate the most reactive ROS, that is hydroxyl radicals ( $\cdot OH$ ) [15], and, ultimately, lipid-derived carbon- and oxygen-centered radicals, as lipid peroxidation (LPO) products. In turn, 6-OHDA quinone triggers a cascade of oxidative reactions finally resulting

in the formation of an insoluble polymeric pigment related to neuromelanin [16,17]. It has been also reported that 6-OHDA inhibits complexes I and IV of the mitochondrial respiratory chain [18,19]. The oxidative stress caused by  $\cdot\text{OH}$  generated during 6-OHDA autoxidation is suggested as a major causal factor of its neurotoxicity [20].

Proteins are major targets for ROS and secondary by-products of oxidative stress, and ROS-induced protein modifications can lead to unfolding or alteration of protein structure. Protein carbonylation (PCO) is an irreversible oxidative damage, that often leads to formation of high-molecular-weight aggregates, which are resistant to degradation and accumulated as damaged or unfolded proteins [21]. PCO can take place through different oxidative pathways. ROS can react (i) directly with the protein, or (ii) with molecules, such as sugars and lipids, generating in turn reactive carbonyl species (RCS), which then react with protein. RCS, generated by peroxidation of polyunsaturated fatty acids, induce PCO, playing a major role in the etiology and/or progression of several human illnesses, including neurodegenerative disorders associated with the deposition of protein aggregates in tissues [22,23]. Some proteins are more susceptible than others to oxidative stress. For example, human brain copper-zinc superoxide dismutase (SOD1) has been proven to be a major target of oxidative damage in PD and AD [24].

In the last decade, nitron spin traps,  $\alpha$ -phenyl-*N*-*tert*-butylnitron (PBN) and structurally related compounds (Fig. 1), which react covalently with short-lived free radicals, such as  $\cdot\text{OH}$ , have shown efficacy in a variety of animal models of CNS injury [25-27], proving to be effective in ischemic stroke models (especially the PBN disodium 1,3-disulfonate salt derivative, NXY-059) [28] and aging [29,30]. Incorporating the nitron functionality in a ring (cyclic PBN analogs) did strongly increase potency relative to PBN against oxidative injury and cell toxicity, as a result of favorable changes in the HOMO/LUMO energy levels and greater accessibility of the nitron double bond [26,31-34]. A number of 3,3-dimethyl-3,4-

dihydroisoquinoline 2-oxides (**1**, Fig. 1), 3,3-dimethyl-4,5-dihydro-3*H*-2-benzazepine 2-oxides (**2**), and their spiro C-3 cycloalkyl analogs, have been found out as inhibitors of lipid peroxidation (LPO). Electron spin resonance (ESR) spectroscopy has clearly demonstrated that cyclic nitrones **1** and **2** are able to trap radicals, forming more stable spin adducts than PBN [33,34]. In particular, chloro-substituted 2-benzazepine nitrones (**2**, X = 8-Cl or 7,9-(Cl)<sub>2</sub>) proved to be potent radical scavengers in both lipid and apoprotein fractions of low density lipoproteins [33]. Moreover, structure-activity relationship (SAR) studies have highlighted the following main factors increasing their *in vitro* inhibitory potency: (i) the presence of a second antioxidant functionality, such as *o,o'*-dimethylphenol (e.g., X = 6,8-(CH<sub>3</sub>)<sub>2</sub>-7-OH in **2**); (ii) lipophilicity increments, as resulted from expansion of the nitron-containing ring (2-benzazepine versus isoquinoline), replacement of the *gem*-dimethyl group at the C-3 position with a spirocyclohexyl moiety and/or hydrophobic X substituents on the fused benzene ring.

[insert Fig. 1]

Recently, we have prepared a number of 2-benzazepine nitron congeners (compounds **3-12**) through an efficient synthesis (Fig. 2) [35] and studied their neuroprotective properties, as assessed by the ability to scavenge  $\cdot\text{OH}$ , generated during 6-OHDA autoxidation, and to inhibit 6-OHDA-induced lipid peroxide formation (LPO), as assessed by monitoring the formation thiobarbituric acid reactive substances (TBARS), and protein oxidative damage, as indicated by the increase of protein carbonyl (PCO) content, in rat brain mitochondria. Herein we report the results from this neurobiological investigation, followed by structure-activity relationships studies to analyze the data. Chiral compounds **3**, **5**, **8-10** and **12** were tested as

racemic mixtures, the investigation of enantioselective effects being out of the scope of this work.

[insert Fig. 2]

## 2. Materials and Methods

### 2.1. Chemicals

PBN, 6-OHDA hydrobromide, terephthalic acid, thiobarbituric acid, butylated hydroxytoluene crystalline, 2,4-dinitrophenylhydrazine hydrochloride, desferrioxamine, 1,1,3,3-tetramethoxypropane, 5,5'-dithiobis-(2-nitrobenzoic acid), sodium dodecylsulfate (SDS), EDTA, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Guanidine hydrochloride was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). The water used for the preparations of solutions was of 18.2 M $\Omega$  (Milli-RiOs/Q-A10 grade, Millipore Corp., Bedford, MA, USA). All remaining chemicals used were of analytical grade and were purchased from Fluka Chemie AG (Buchs, Switzerland).

### 2.2. Synthesis of 4,5-dihydro-3H-2-benzazepine N-oxide derivatives

3,3-Dimethyl-4,5-dihydro-3H-2-benzazepine N-oxide derivatives and their spiro C-3-cycloalkane analogs were prepared through an efficient synthesis reported by some of us [35]. First, 2,3,4,5-tetrahydro-1H-2-benzazepines were easily synthesized in two steps from readily available imines. They were then converted, with moderate-to-high yields, into the 2-benzazepine nitrones *via* hydrogen peroxide oxidation in the presence of catalytic amounts of sodium tungstate, under mild conditions. 5-Methyl-4,5-dihydrospiro[2-benzazepine-3,1'-cyclopentane] 2-oxide (**9**), 5-methyl-4,5-dihydrospiro[2-benzazepine-3,1'-cyclohexane] 2-oxide (**10**), and 1,5-dimethyl-4,5-dihydrospiro[2-benzazepine-3,1'-cyclohexane] 2-oxide (**12**)

have been reported earlier [35]. Compounds **4**, **9** and **12** were prepared and tested as hydrochlorides.

### 2.2.1. Homoallylamines

*2-Methyl-N-(phenylmethyl)pent-4-en-2-amine* (for **3**), *2,4-dimethyl-N-(phenylmethyl)pent-4-en-2-amine* (for **4**), *2-methyl-N-(1-phenylethyl)pent-4-en-2-amine* (for **5**), *2,4-dimethyl-N-(1-phenylethyl)pent-4-en-2-amine* (for **6**), *N-(diphenylmethyl)-2,4-dimethylpent-4-en-2-amine* (for **7**), *3,5-dimethyl-N-(phenylmethyl)hex-5-en-3-amine* (for **8**), and *1-(2-methylprop-2-en-1-yl)-N-(phenylmethyl)cyclohexanamine* (for **11**) were obtained according to reported procedures [36-38] from the corresponding imines and allyl(methyl)lithium bromide.

### 2.2.2. 2,3,4,5-Tetrahydro-1H-2-benzazepine

*3,3,5-Trimethyl-2,3,4,5-tetrahydro-1H-2-benzazepine* (for **3**), *3,3,5,5-tetramethyl-2,3,4,5-tetrahydro-1H-2-benzazepine* (for **4**), *1,3,3,5-tetramethyl-2,3,4,5-tetrahydro-1H-2-benzazepine* (for **5**), *1,3,3,5,5-pentamethyl-2,3,4,5-tetrahydro-1H-2-benzazepine* (for **6**), *3,3,5,5-tetramethyl-1-phenyl-2,3,4,5-tetrahydro-1H-2-benzazepine* (for **7**), *3-ethyl-3,5,5-trimethyl-2,3,4,5-tetrahydro-1H-2-benzazepine* (for **8**), and *5,5-dimethyl-1,2,4,5-tetrahydrospiro[2-benzazepine-3,1'-cyclohexane]* (for **11**) were obtained according to already described methods [31, 39]. Selected spectral and physical data for 2-benzazepines are given below.

*3,3,5,5-Tetramethyl-2,3,4,5-tetrahydro-1H-2-benzazepine* (for **4**). Pale-yellow oil, 55% yield. bp 104-7 °C/2 torr; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 1.22 (s, 6H), 1.41 (s, 6H), 1.43 (br. s, 1H), 1.81 (s, *J* = 14.9 Hz, 1H), 1.82 (d, *J* = 14.9 Hz, 1H), 4.03 (s, 2H), 7.01 (dd, *J* = 7.6, 2.1 Hz, 1H), 7.06 (dt, *J* = 7.6, 2.6 Hz, 1H), 7.17 (dt, *J* = 7.6, 2.1 Hz, 1H), 7.34 (dd, *J* = 7.6, 2.6 Hz,

1H) ppm; ESI-MS  $m/z$ : 204 (MH)<sup>+</sup>. Anal. Calcd. for C<sub>14</sub>H<sub>21</sub>N: C, 82.70; H, 10.41; N, 6.89.

Found: C, 82.76; H, 10.34; N, 6.90.

*1,3,3,5-Tetramethyl-2,3,4,5-tetrahydro-1H-2-benzazepine* (for **5**). Colourless oil, 42% yield.

bp 100-1 °C/3 torr; isomer ratio 1:1.5, <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz), major isomer: δ 1.06 (s, 3H), 1.26 (dd,  $J = 13.9, 11.5$  Hz, 1H), 1.39 (s, 3H), 1.40 (d,  $J = 7.3$  Hz, 3H), 1.54 (d,  $J = 6.8$  Hz, 3H), 1.60 (d,  $J = 13.9$  Hz, 1H), 3.37 (dq,  $J = 11.5, 7.3$  Hz, 1H), 7.19-7.34 (m, 4H) ppm;

minor isomer: δ 1.15 (s, 3H), 1.17 (s, 3H), 1.41 (d,  $J = 7.3$  Hz, 3H), 1.53 (d,  $J = 6.8$  Hz, 3H), 1.74 (dd,  $J = 13.9, 10.3$  Hz, 1H), 1.83 (dd,  $J = 13.9, 3.7$  Hz, 1H), 3.43 (m,  $J = 10.3, 7.3, 3.7$  Hz, 1H), 4.41 (q,  $J = 6.8$  Hz, 1H), 7.19-7.34 (m, 4H) ppm; ESI-MS  $m/z$ : 204 (MH)<sup>+</sup>. Anal.

Calcd. for C<sub>14</sub>H<sub>21</sub>N: C, 82.70; H, 10.41; N, 6.89. Found: C, 82.66; H, 10.39; N, 6.85.

*1,3,3,5,5-Pentamethyl-2,3,4,5-tetrahydro-1H-2-benzazepine* (for **6**). Pale-yellow oil, 50%

yield. bp 103-4 °C/3 torr; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 1.24 (s, 3H), 1.25 (s, 3H), 1.41 (s, 3H), 1.49 (s, 3H), 1.51 (d,  $J = 6.7$  Hz, 3H), 1.60 (d,  $J = 15.0$  Hz, 1H), 2.12 (d,  $J = 15.0$  Hz, 1H), 4.46 (d,  $J = 6.7$  Hz, 1H), 7.15 (dt,  $J = 7.3, 1.6$  Hz, 1H), 7.19 (dt,  $J = 7.3, 1.6$  Hz, 1H), 7.27 (dd,  $J = 7.3, 1.6$  Hz, 1H), 7.38 (dd,  $J = 7.3, 1.6$  Hz, 1H) ppm; ESI-MS  $m/z$ : 218 (MH)<sup>+</sup>.

Anal. Calcd. for C<sub>15</sub>H<sub>23</sub>N: C, 82.89; H, 10.67; N, 6.44. Found: C, 82.95; H, 10.60; N, 6.45.

*3-Ethyl-3,5,5-trimethyl-2,3,4,5-tetrahydro-1H-2-benzazepine* (for **8**). Pale-yellow oil, 66%

yield. bp 131-2 °C/5 torr; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 0.93 (t,  $J = 7.3$  Hz, 3H), 1.16 (s, 3H), 1.42 (s, 3H), 1.46 (s, 3H), 1.54 (m, 2H), 1.74 (d,  $J = 15.1$  Hz, 1H), 1.86 (d,  $J = 15.1$  Hz, 1H), 4.05 (s, 1H), 7.04 (dd,  $J = 7.5, 0.9$  Hz, 1H), 7.20 (t,  $J = 7.5, 1.4$  Hz, 1H), 7.37 (dd,  $J = 7.5, 1.4$  Hz, 1H), 7.39 (td,  $J = 7.5, 0.9$  Hz, 1H) ppm; ESI-MS  $m/z$ : 218 (MH)<sup>+</sup>. Anal. Calcd. for

C<sub>15</sub>H<sub>23</sub>N: C, 82.89; H, 10.67; N, 6.44. Found: C, 82.92; H, 10.69; N, 6.45.

*5,5-Dimethyl-1,2,4,5-tetrahydrospiro[2-benzazepine-3,1'-cyclohexane]* (for **11**). Viscous

yellow oil, 85% yield. bp 139-42 °C/2 torr; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 1.42 (s, 6H), 1.25-

1.75 (m, 10H), 1.79 (s, 2H), 4.01 (s, 2H), 6.89-7.35 (m, 4H) ppm; ESI-MS  $m/z$ : 244 (MH)<sup>+</sup>.  
Anal. Calcd. for C<sub>17</sub>H<sub>25</sub>N: C, 83.95; H, 10.29; N, 5.76. Found: C, 84.24; H, 9.95; N, 5.81.

### 2.2.3. Oxidation of benz-2-azepines to nitrones (**3-8**, **11**): Typical procedure.

To a solution of 50 mmol of benz-2-azepine and Na<sub>2</sub>WO<sub>4</sub>·2H<sub>2</sub>O (0.83 g, 2.5 mmol) in acetone–water mixture (9:1, v/v, 100 mL), 50% H<sub>2</sub>O<sub>2</sub> (12 mL, 200 mmol) was added dropwise at 0 °C (0.5 hour). The resulting mixture was stirred at room temperature for 24- 96 hours (TLC monitoring). After the reaction was completed, the reaction mixture was poured into water (300 mL) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (5×30 mL). The combined organic extracts were dried over MgSO<sub>4</sub> and concentrated *in vacuo*. Crystallization of the resulting solids from hexane–ethyl acetate mixture gave white crystals of nitrones (**3-8**, **11**). In case of compounds **3**, **5**, **8** the oily residues were purified by column chromatography on Al<sub>2</sub>O<sub>3</sub> (using hexane–ethyl acetate, 5/1, v/v, as eluent). Slowly crystallizing nitrones (**3**, **6**, **8**, **11**) were converted into hydrochlorides by mixing their ether solutions (1 g of nitrone in 50 ml of dry diethyl ether) with saturated HCl/Et<sub>2</sub>O solution until pH 5 was achieved. Resulting white precipitates were filtered-off, washed with absolute ether and dried on air (90-95 % yields).

**3,3,5-Trimethyl-4,5-dihydro-3H-2-benzazepine 2-oxide (3)**. White crystals, 75% yield. mp 71-73 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 1.43 (d,  $J = 7.2$  Hz, 3H), 1.53 (s, 3H), 1.65(s, 3H), 1.99 (dd,  $J = 1.9, 15.0$  Hz, 1H), 2.12 (dd,  $J = 9.4, 15.0$  Hz, 1H), 3.13 (m, 1H), 7.14-7.30 (m, 4H), 7.91 (s, 1H); EI-MS (70 eV)  $m/z$  (rel. intensity): 203 (32, M<sup>+</sup>), 144 (16), 132 (100), 131 (26), 130 (28), 129 (16), 128 (19), 115 (29), 104 (37), 77 (22); IR (KBr, ν cm<sup>-1</sup>) 1529 (C=N). Anal. Calcd. for C<sub>13</sub>H<sub>17</sub>NO: C, 76.81; H, 8.43; N, 6.89. Found: C, 76.65; H, 8.51; N, 6.81.

**3,3,5,5-Tetramethyl-4,5-dihydro-3H-2-benzazepine 2-oxide (4)**. White crystals, 55% yield. mp 51-2 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 1.49 (s, 3H), 1.72 (s, 3H), 2.29 (s, 2H), 7.44 (td,  $J = 7.5, 1.2$  Hz, 1H), 7.52 (bd,  $J = 7.5$  Hz, 1H), 7.62-7.71 (m, 2H), 9.09 (s, 1H); EI-MS (70 eV)

*m/z* (rel. intensity): 217 (35, M<sup>+</sup>), 161 (22), 146 (39), 145 (29), 144 (55), 143 (16), 130 (14), 129 (28), 128 (100), 115 (31); IR (KBr,  $\nu$  cm<sup>-1</sup>) 1545 (C=N). Anal. Calcd. for C<sub>14</sub>H<sub>19</sub>NO: C, 77.38; H, 8.81; N, 6.45. Found: C, 77.42; H, 8.85; N, 6.66.

*1,3,3,5-Tetramethyl-4,5-dihydro-3H-2-benzazepine 2-oxide (5)*. White crystals, 66% yield. mp 69-71 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  0.82 (s, 3H), 1.23 (d, *J* = 6.8 Hz, 3H), 1.30 (s, 3H), 1.86 (dd, *J* = 12.2, 14.0 Hz, 1H), 2.30 (s, 3H), 2.45 (dd, *J* = 6.8, 14.0 Hz, 1H), 2.98 (m, 1H), 7.27-7.31 (m, 4H); EI-MS (70 eV) *m/z* (rel. intensity): 217 (48, M<sup>+</sup>), 175 (22), 160 (23), 158 (40), 146 (100), 145 (54), 144 (56), 143 (31), 128 (26), 91 (29); IR (KBr,  $\nu$  cm<sup>-1</sup>) 1542 (C=N). Anal. Calcd. for C<sub>14</sub>H<sub>19</sub>NO: C, 77.38; H, 8.81; N, 6.45. Found: C, 77.52; H, 8.75; N, 6.43.

*1,3,3,5,5-Pentamethyl-4,5-dihydro-3H-2-benzazepine 2-oxide (6)*. White crystals (hydrochloride), 70% yield. mp 165-73 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.21 (s, 6H), 1.30 (s, 6H), 2.25 (s, 2H), 2.44 (s, 3H), 7.21-7.30 (m, 1H), 7.28-7.33 (m, 2H), 7.35-7.38 (m, 1H); EI-MS (70 eV) *m/z* (rel. intensity): 231 (24, M<sup>+</sup>), 160 (59), 159 (22), 158 (100), 157 (19), 144 (18), 143 (20), 129 (19), 128 (29), 115 (21); IR (KBr,  $\nu$  cm<sup>-1</sup>) 1555 (C=N). Anal. Calcd. for C<sub>15</sub>H<sub>21</sub>NO: C, 77.88; H, 9.15; N, 6.05. Found: C, 77.81; H, 9.30; N, 6.18.

*3,3,5,5-Tetramethyl-1-phenyl-4,5-dihydro-3H-2-benzazepine 2-oxide (7)*. White crystals, 73% yield. mp 97-9 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.20 (s, 6H), 1.38 (s, 6H), 2.23 (s, 2H), 7.2-7.7 (m, 9H); EI-MS (70 eV) *m/z* (rel. intensity): 293 (56, M<sup>+</sup>), 251 (22), 237 (19), 222 (70), 221 (32), 220 (100), 158 (37), 128 (20), 91 (21), 77 (38), 58 (19); IR (KBr,  $\nu$  cm<sup>-1</sup>) 1540 (C=N). Anal. Calcd. for C<sub>20</sub>H<sub>23</sub>NO: C, 81.87; H, 7.90; N, 4.77. Found: C, 81.97; H, 7.77; N, 4.73.

*3-Ethyl-3,5,5-trimethyl-4,5-dihydro-3H-2-benzazepine 2-oxide (8)*. White crystals, 67% yield. mp 81-2 °C (162-9 °C for hydrochloride); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  0.81 (t, *J* = 7.4 Hz, 3H), 1.3 (s, 3H), 1.44 (s, 3H), 1.58 (s, 3H), 1.71 (qd, *J* = 7.4, 14.4 Hz, 1H), 1.83 (qd, *J* = 7.4,

14.4 Hz, 1H), 2.04 (d,  $J = 15.4$  Hz, 1H), 2.27 (d,  $J = 15.4$  Hz, 1H), 7.10 (bd,  $J = 7.6$  Hz, 1H), 7.20 (td,  $J = 7.6, 1.3$  Hz, 1H), 7.26 (td,  $J = 7.6, 1.3$  Hz, 1H), 7.33 (bd,  $J = 7.6$  Hz, 1H), 7.97 (s, 1H); EI-MS (70 eV)  $m/z$  (rel. intensity): 231 (16,  $M^+$ ), 214 (17), 202 (80), 186 (29), 162 (55), 160 (12), 145 (100), 144 (56), 143 (30), 130 (35), 129 (57), 128 (38), 115 (33), 91 (17), 77 (13), 41 (10); IR (KBr,  $\nu$   $\text{cm}^{-1}$ ) 1542 (C=N). Anal. Calcd. for  $\text{C}_{15}\text{H}_{21}\text{NO}$ : C, 77.88; H, 9.15; N, 6.05. Found: C, 77.90; H, 9.21; N, 6.17.

*5,5-Dimethyl-4,5-dihydrospiro[2-benzazepine-3,1'-cyclohexane] 2-oxide (11)*. White crystals (hydrochloride), 50% yield. mp 164-5 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz)  $\delta$  1.36-1.61 (m, 4H), 1.51 (s, 6H), 1.72-1.90 (m, 4H), 2.33-2.43 (m, 2H), 2.41 (s, 2H), 7.43 (td,  $J = 7.6, 1.3$  Hz, 1H), 7.49-7.53 (m, 1H), 7.59-7.66 (m, 2H), 9.13 (s, 1H); EI-MS (70 eV)  $m/z$  (rel. intensity): 257 (11,  $M^+$ ), 240 (100), 198 (26), 145 (17), 144 (16), 143 (12), 130 (15), 129 (28), 128 (33), 115 (16); IR (KBr,  $\nu$   $\text{cm}^{-1}$ ) 1586 (C=N). Anal. Calcd. for  $\text{C}_{17}\text{H}_{23}\text{NO}$ : C, 79.33; H, 9.01; N, 5.44. Found: C, 79.28; H, 9.16; N, 5.38.

### 2.3. Preparation of brain mitochondria

All procedures were carried out in accordance with the NIH *Guide for Care and Use of Laboratory Animals*, and were approved by the Animal Ethics Committee of the Universidad de Santiago de Compostela (Santiago de Compostela, Spain). Male Sprague–Dawley rats weighting 250–300 g were used. The rats were received from the breeder (Animalario de la Universidad de Santiago de Compostela) at least 2 days before sacrifice, and were kept at 22 °C, on a 12:12 light–dark schedule, and with *ad libitum* access to food and water. Animals were stunned with carbon dioxide and killed by decapitation. Brains were immediately removed and washed in ice-cold isolation medium (pH 7.4,  $\text{Na}_2\text{PO}_4/\text{KH}_2\text{PO}_4$  isotonized with sucrose). Brain mitochondria were then obtained by differential centrifugation with minor

modifications to a previously published method [40]. Briefly, after removing blood vessels and pial membranes, the brains were manually homogenized with four volumes (w/v) of the isolation medium. Then, the homogenate was centrifuged in an Avanti J-25 centrifuge (Beckman Instruments, Palo Alto, USA) at  $1,000\times g$  for 5 min at 4 °C. The supernatant was centrifuged at  $12,500\times g$  for 15 min. The mitochondrial pellet was then washed once with isolation medium and recentrifuged under the same conditions. Finally, the mitochondrial pellet was reconstituted in a buffer solution ( $\text{Na}_2\text{PO}_4/\text{KH}_2\text{PO}_4$  isotonized with KCl, pH 7.4) and stored in aliquots under liquid nitrogen. The protein concentration of mitochondrial preparations was determined according to the method of Markwell *et al.* [41], using bovine serum albumin (BSA) as the standard.

#### 2.4. Fluorimetric monitoring of hydroxyl radical formation

The hydroxyl radical ( $\cdot\text{OH}$ ) formation was monitored fluorimetrically using a previously reported method [14,40,42,43], in which terephthalic acid (THA) is used as a dosimeter for the detection of  $\cdot\text{OH}$  in vitro. A luminescence spectrometer Model LS50B (Perkin-Elmer, Norwalk, CT, USA) was used. The cuvette holder was thermostatically maintained at 37 °C and the sample continuously stirred with a magnetic stirrer.

A buffer solution ( $\text{Na}_2\text{PO}_4/\text{KH}_2\text{PO}_4$  isotonized with KCl, pH 7.4) containing 10 mM terephthalic acid was incubated for 5 min to reach the working temperature. Then, the test 2-benzazepine nitron (final concentration range from 5  $\mu\text{M}$  to 1 mM) or water was incorporated into the incubation followed by addition of either 6-OHDA (10  $\mu\text{M}$ ) or 1 mM KCl (pH 2.0). All the reported concentrations are final concentrations in the incubation mixture. Monitoring of  $\cdot\text{OH}$  formation was immediately initiated and maintained for the subsequent 10 min. The excitation and emission wavelengths used were 312 and 426 nm,

respectively. Fluorescence measurements were all relative to the initial reading and the peak of relative fluorescence ( $\Delta F_{\max}$ ) used to express the amount of the produced  $\cdot\text{OH}$ .

The test compound concentration that inhibited  $\cdot\text{OH}$  formation by 50% ( $\text{IC}_{50}$ ) was calculated using the program Origin<sup>®</sup> v. 6.0 (Microcal Software Inc., Northampton, MA, USA) from a concentration-response curve generated with seven different concentrations of the 2-benzazepine nitronone (Table 1).

### 2.5. Lipid peroxidation assay

The inhibitory effects of the test nitronones on the lipid peroxidation (LPO) were assessed by monitoring the formation of thiobarbituric acid reactive substances (TBARS), using a slightly modified spectrophotometric method [40].

A brain mitochondrial preparation (1 mg protein/ml), in a  $\text{Na}_2\text{PO}_4/\text{KH}_2\text{PO}_4$  buffer (pH 7.4) isotonized with KCl, was incubated at 37 °C for 5 min to reach the working temperature. Then, the test nitronone (concentrations ranging from 5  $\mu\text{M}$  to 1 mM) or water was incorporated into the incubation, followed by addition of 6-OHDA (10  $\mu\text{M}$ ) or 1mM KCl (pH 2.0), and the mixture incubated for exactly 20 min. Butylated hydroxytoluene (20  $\mu\text{M}$ ) and desferrioxamine (20  $\mu\text{M}$ ) were immediately added, in order to prevent amplification of the lipid peroxidation during the assay. All the above reported concentrations are final concentrations in the incubation medium.

An aliquot of the sample (200  $\mu\text{l}$ ) was treated with SDS (8.1%, w/v), followed by addition of acetic acid (20%), and the mixture vortexed for 1 min. Then, thiobarbituric acid (TBA, 0.8%) was added and the resulting mixture incubated at 95 °C for 60 min. After cooling to room temperature, 3 ml of *n*-butanol were added and the mixture shaken vigorously. After centrifugation at 2,500 $\times g$  for 10 min, the absorbance of the supernatant (organic layer) was

measured at 532 nm using an Ultrospec III spectrophotometer (Pharmacia Biotech, Uppsala, Sweden).

For calibration, a standard curve (5–150 nM) was generated using the malondialdehyde (MDA), prepared via acid-catalyzed hydrolysis ( $\text{H}_2\text{SO}_4$ ; 1.5%, v/v) of 1,1,3,3-tetraethoxypropane. The protein concentration of the sample was determined according to a previously reported method [41], using BSA as the standard. The  $\text{IC}_{50}$  values are listed in Table 2.

## 2.6. *Assessment of protein carbonyl content*

The ability of the examined cyclic nitrones to inhibit the protein oxidative damage caused by the autoxidation of 10  $\mu\text{M}$  6-OHDA in the rat brain mitochondrial preparation was assayed by determining the content of protein carbonyls (PCOs), through a modified published spectrophotometric method [40]. Brain mitochondria (1 mg protein/ml) were incubated in  $\text{Na}_2\text{PO}_4/\text{KH}_2\text{PO}_4$  buffer (pH 7.4, isotonized with KCl), at 37 °C for 5 min. Then, the test nitrone (final concentrations ranging from 5  $\mu\text{M}$  to 1 mM) or water was incorporated into the incubation, followed by 6-OHDA (10  $\mu\text{M}$ ) or 1mM KCl (pH 2.0), and the mixture incubated for exactly 20 min.

An aliquot of the sample (200  $\mu\text{l}$ ) was then immediately submitted to protein precipitation, by adding of trichloroacetic acid (TCA 20%, w/v), followed by centrifugation at 15,000 $\times$ g for 5 min. The resulting pellet was reconstituted in 0.5 M NaOH with sonication (Branson Sonic Corp., Danbury, CT, USA) for 5 s. Then, 10 mM 2,4-dinitrophenylhydrazine in 2M chloric acid was added and the mixture incubated at room temperature for 1 h, in darkness with continuous agitation. After the addition of TCA (20%, w/v), the mixture was centrifuged at 15,000 $\times$ g for 5 min. The resulting pellet was washed twice with ethyl acetate:EtOH (1:1, v/v).

Then, the washed pellet was reconstituted with 6M guanidine in a 20 mM KH<sub>2</sub>PO<sub>4</sub> buffer (pH 2.3) and centrifuged at 15,000×g for 5 min. The absorbance of the resulting solution was measured at 370 nm.

The carbonyl content was calculated from the absorbance data, using as absorption coefficient for dinitrophenylhydrazone  $\epsilon = 22,000 \text{ M}^{-1} \text{ cm}^{-1}$ , and expressed as nmol carbonyls/mg protein. Because of the numerous washing steps, protein content in the final pellet was estimated on an HCl blank pellet processed simultaneously, using a BSA standard curve in 6 M guanidine, and reading the absorbance at 280 nm [44]. The IC<sub>50</sub> values are listed in Table 2.

### 2.7. *Statistical Analysis*

Data are expressed as mean  $\pm$  SEM. Differences between means were statistically evaluated using the one-way ANOVA followed by the Bonferroni test. Normality of populations and homogeneity of variances was tested before each ANOVA. The accepted level of significance in all cases was  $P < 0.05$ .

### 2.8. *Determination of lipophilicity parameters by RP-HPLC*

The lipophilicity of the 2-benzazepine nitron derivatives was determined by a reversed-phase HPLC method [45,46]. Compounds were dissolved in MeOH at 0.25 mg/ml, injected onto a Symmetry C18 column (15 cm  $\times$  3.9 mm i.d., 5  $\mu\text{m}$  particles) from Waters Assoc. (Milford, MA, U.S.A.), and retention data measured at regular increments of the volume fraction of methanol in pH 5.0 acetate buffer (40 mM). All the measurements were made at temperature of  $25 \pm 1 \text{ }^\circ\text{C}$ , flow-rate of 1.0 ml/min and at 254 nm wavelength, on a Waters HPLC 1525

multisolvent delivery system, equipped with a Waters 2487 variable wavelength UV detector (Waters Assoc., Milford, MA, U.S.A.).

Capacity factors ( $k'$ ) of each compound at different mobile phase compositions (0.05-increments of MeOH volume fraction, ranging between 0.7 and 0.3) were calculated as:  $k' = (t_R - t_0)/t_0$ , where  $t_R$  is the retention time of the solute and  $t_0$  is the column dead time, measured as the elution time of MeOH. In all the cases, the  $\log k'$  values increased with decreasing MeOH volume fraction. Linear regression analysis ( $r^2 > 0.96$ ) was performed on at least five data points (the lowest MeOH concentrations) for each compound and the linear relationship extrapolated to 100% aqueous mobile phase to yield  $\log k'_w$  values.

Lipophilicity was also computationally assessed with the ACDLabs software, release 9.0 (Advanced Chemistry Development, Inc., Toronto, Canada). Both, computational and experimental lipophilicity values are listed in Table 2.

### 3. Results

The effects of the examined 2-benzazepine nitrones (Fig. 2) on the  $\cdot\text{OH}$  generated during 6-OHDA (10  $\mu\text{M}$ ) autoxidation were assessed by measuring the relative fluorescence after 10 min incubation time, using THA as a chemical dosimeter [14,40,42,43], and the maximal relative fluorescence ( $\Delta F_{\text{max}}$ ) was taken as a measure of  $\cdot\text{OH}$  production. Fig. 3 shows a typical recording of  $\cdot\text{OH}$  formation in the absence (control) and presence of the reference compound PBN, the most simple 2-benzazepine 2-oxide within the series (**3**), taken as the positive control, and the spiro C-3 cyclopentyl analog (**9**), behaving as the most effective compound in this assay. The  $\text{IC}_{50}$  values determined for the whole nitrone set are listed in Table 1.

[insert Fig. 3 and Table 1]

In line with expectations, all the 7-membered ring nitrones inhibited 6-OHDA-dependent  $\cdot\text{OH}$  formation more potently than PBN. The analog **9**, ( $\pm$ )-5-methyl-4,5-dihydrospiro[2-benzazepine-3,1'-cyclopentane] 2-oxide, proved to be the most active one ( $\text{IC}_{50} = 7 \mu\text{M}$ ), displaying more than one hundred-fold improvement over PBN. A modest, but significant, increase of the  $\cdot\text{OH}$ -scavenging activity was observed in compounds bearing 5-*gem*-dimethyl group (**4**, **6** and **11**) compared with the respective mono-methyl derivatives (**3**, **5** and **10**), whereas adding a methyl group to the nitrone C-1 (**5**, **6** and **12**), and to a lesser extent a phenyl group (**7**), decreases the reactivity of the nitrone group most likely because of its limited steric accessibility.

6-OHDA-induced oxidative stress, producing significant increase of LPO product level and protein damage, is believed to be involved in neuronal cell death related to PD. Using this

model, we studied the effects of the 2-benzazepine nitrones **3-12** on 6-OHDA-induced oxidative damage in rat brain mitochondrial membrane homogenates as previously described [40], assessing lipid peroxidation (LPO), as measured by the formation of thiobarbituric acid reactive substances (TBARS), and protein oxidative damage, as revealed by the protein carbonylation (PCO).

With the exceptions of compounds **5** and **12**, which did not attain 50% LPO inhibition in the 5-1000  $\mu\text{M}$  concentration range, all the tested benzazepine *N*-oxides provoked marked reduction in the level of TBARS, showing inhibitory potencies higher than that of PBN. Inhibition was concentration dependent (Fig. 4) and  $\text{IC}_{50}$  values varied with different nitrones (Table 2).

[insert Fig. 4 and Table 2]

The most simple cyclic nitronone assayed (**3**) displayed more than 20-fold increase of LPO inhibition potency over PBN, but they were once again the spiro C-3 cycloalkyl analogs which showed the most remarkable effects with  $\text{IC}_{50}$  values of 20-43  $\mu\text{M}$  (**9-11**). Among them, compound **11**, which bears 5-*gem*-dimethyl and C-3 spirocyclohexyl groups, behaved as the most potent one. Nitrones **5** and **12** lost LPO inhibition activity, likely due to the steric effect of  $\text{CH}_3$   $\alpha$  to *N*-oxide group, such an unfavorable effect being partially reversed in compounds **6** ( $\text{R}^1 = \text{methyl}$ ) and **7** ( $\text{R}^1 = \text{phenyl}$ ), thanks to the potency-increasing effects of the *gem*-dimethyl group at the C-5 position. A plot of  $\text{pIC}_{50}$  ( $-\log \text{IC}_{50}$ ) values in TBARS assay vs  $\cdot\text{OH}$  scavenging activity (Fig. 5) shows that the potency of the 2-benzazepine nitrones in inhibiting LPO is reasonably correlated with their  $\cdot\text{OH}$  scavenging ability. To allow SAR information to be retained, we used a truncated  $\text{pIC}_{50}^{\text{LPO}}$  value of 3.00 for

compounds **5** and **12** ( $IC_{50} > 1$  mM), and obtained the following linear eq. 1, which explains more than 70% of the variance in y-data:

$$pIC_{50}^{LPO} = 1.02 (\pm 0.23) pIC_{50}^{OH} - 0.61 (\pm 1.00) \quad (\text{eq. 1})$$

$$n = 10, r^2 = 0.711, s = 0.336, F = 19.6$$

where  $n$  represents the number of data points,  $r^2$  the squared correlation coefficient,  $s$  the standard deviation of the regression equation,  $F$  the statistical significance of fit; 95% confidence intervals of the regression coefficients are given in parentheses.

[insert Fig. 5]

With respect to the effect of 6-OHDA on the oxidative status of mitochondrial proteins, herein assayed by measuring the extent of PCO, PBN displayed a very low inhibitory activity ( $IC_{50} = 11.3$  mM). In contrast, the addition to the incubation of the 2-benzazepine nitrones, with the only exception of compound **5**, caused significant reduction in carbonyl content in rat brain mitochondrial proteins (Table 2). The nitrone derivative **3** inhibited PCO with an  $IC_{50}$  value of 97  $\mu$ M, but compounds **4**, **9** and **11** showed a two-fold increase of the inhibitory potency over **3**. PCO inhibition effects quite similar to those measured on LPO, albeit not linearly correlated to them, were observed. As a matter of fact, like in LPO inhibition, 5-*gem*-dimethyl derivatives were more potent inhibitors than the corresponding 5-methyl ones (compare **4**, **6** and **11** with **3**, **5** and **10**, respectively), as well as  $R^1$ -methyl bearing 2-benzazepine nitrones (**5**, **6** and **12**) loose antioxidant activity compared to the corresponding  $R^1$ -desmethyl compounds (**3**, **4** and **10**), but in contrast with the LPO/TBARS results, in the PCO assay the spiro C-3 cyclohexyl compounds **10** and **11** did not show any remarkable

increase of potency compared with the corresponding 3-*gem*-dimethyl analogs **3** and **4**, respectively. Linear regression between pIC<sub>50</sub> value in PCO assay (a truncated pIC<sub>50</sub><sup>PCO</sup> value of 3.00 was used, in this case, for compound **5**) and 'OH-decreasing ability yielded the following linear eq. 2, statistically poorer than eq. 1:

$$\text{pIC}_{50}^{\text{PCO}} = 0.71 (\pm 0.19) \text{pIC}_{50}^{\text{OH}} + 0.85 (\pm 0.19) \quad (\text{eq. 2})$$

$$n = 10, r^2 = 0.640, s = 0.276, F = 14.2$$

Taking into account the results obtained by others [31-34], who highlight a major role of lipophilicity in modulating LPO inhibition by cyclic nitrones, we determined the relative lipophilicity of the 2-benzazepine nitrones by a well-established reversed-phase (RP)-HPLC technique ( $\log k'_w$ ) [47], using previously reported methods [45,46]. A comparison between the experimental parameters  $\log k'_w$  (Table 2) and octanol-water partition coefficients calculated by the ACDLabs software ( $\text{clog } P$ ) showed that a linear relation between the two sets of lipophilicity parameters holds up to  $\text{clog } P$  of *ca.* 3.5, a value beyond which  $\log k'_w$  no longer increases. This should be not surprising, since the ACDLabs calculation system, as well as other  $\log P$  fragmental calculation methods, may fail in lipophilicity prediction either because of the high uncertainty of the estimated fragmental  $\log P$  values of the nitrone group and connected atoms and/or because the highest  $\log P$  values are too close or above the upper limit for precise partition coefficient measurements, which constitute the basis of the  $\log P$  calculation fragmental system. The spread of the measured  $\log k'_w$  parameters is rather limited (1.14 log units), and most of the examined compounds (**5-8**, **10-12**) varied in just 0.2  $\log k'_w$  units, that is a too small range to be a matter of physicochemical interpretation. Nonetheless, within the limits of the small set examined, when comparing  $\log k'_w$  values among themselves, some significant findings can be highlighted. Thus, the addition of the

second CH<sub>3</sub> group at C-5 leads to a small (compare **4** with **3**) to nil (compare **6** with **5**) increase of lipophilicity. The increment of log  $k'_w$  by about one unit caused by 1-methyl group (**5** versus **3**), and to a lesser extent by 1-phenyl group (**7** versus **4**), should account for the decrease in the water-accessible surface area of the polar N<sup>+</sup>-O<sup>-</sup> group, besides the additive hydrophobic effect of the R<sup>1</sup>-substituents.

Investigating the role of lipophilicity in inhibiting TBARS formation in brain mitochondria, a trend was observed which suggested a bilinear relationship [48] between pIC<sub>50</sub><sup>LPO</sup> and log  $k'_w$ . LPO inhibitory potency of 2-benzazepine nitrones increases as the log  $k'_w$  increases from 2.41 for **3** to 3.05 for **9**; when log  $k'_w$  surpasses the value of 3.37, the potency no longer increases but steeply declines with further increase of the log  $k'_w$ . Nonlinear regression between pIC<sub>50</sub><sup>LPO</sup> and log  $k'_w$  did not afford any statistically valid quantitative SAR, whereas including in eq. 1 the lipophilicity parameter as a second independent variable, the following two-parameter equation was obtained, which explains 90% of the variance in the LPO inhibition data:

$$\text{pIC}_{50}^{\text{LPO}} = 1.18 (\pm 0.23) \text{pIC}_{50}^{\text{OH}} + 0.70 (\pm 0.19) \log k'_w - 3.61 (\pm 1.04) \quad (\text{eq. 3})$$

$$n = 10, r^2 = 0.900, s = 0.211, F = 31.5$$

A plot of the measured vs calculated pIC<sub>50</sub><sup>LPO</sup> values is illustrated in Fig. 5. Eq. 3 shows that the inhibition potency of 6-OHDA-induced TBARS formation in rat brain mitochondria primarily depends upon the •OH scavenging activity, but significantly increases with increasing lipophilicity, as accounted for by the log  $k'_w$  parameter. The introduction of clog  $P$  instead of log  $k'_w$  proved to be not statistically significant. Addition of log  $k'_w$  (and clog  $P$  as well) to eq. 2, which describes the inhibition potency against PCO (pIC<sub>50</sub><sup>PCO</sup>), did not result in

any statistical improvement, suggesting that lipophilicity does not play a role in inhibiting PCO as important as in inhibiting formation of lipid peroxide products.

## 5. Discussion

There is growing evidence indicating that oxidative stress, which results in significant increase of lipid peroxidation products and protein oxidative damage, is a key contributor to the pathogenesis of several neurodegenerative diseases, including PD [14, 49-51]. In this study, we used 6-OHDA, a neurotoxin producing experimental model of PD [52], to generate in vitro  $\cdot\text{OH}$  and to cause LPO and PCO [51], and investigated a series of new seven-membered cyclic analogs of the spin trap PBN, namely 3,3-dimethyl-4,5-dihydro-3*H*-2-benzazepine nitrones and their spiro C-3 cycloalkyl analogs, for their ability to protect rat brain mitochondria against oxidative injury.

Autoxidation of 6-OHDA is an important source of intracellular ROS. Under physiological conditions, it is rapidly oxidized affording formation of  $\text{H}_2\text{O}_2$ ,  $\cdot\text{OH}$ , and the corresponding *p*-quinone [53]. The formation of the hazardous  $\cdot\text{OH}$  during the autoxidation of 6-OHDA has been proven to occur without involvement of ferrous ion, or any other transition metal ion, in Fenton-type reactions [54]. The inhibition of the 6-OHDA-induced oxidative damage by our 2-benzazepine nitrones should primarily stem from their ability to trap radical species, as previously shown by others through ESR studies on strictly related analogs [33]. Our data from a chemical fluorimetric assay using THA as  $\cdot\text{OH}$  dosimeter [42] indicate that compounds **3-12** reduce, more efficiently than does PBN, 6-OHDA-induced  $\cdot\text{OH}$  production (Table 1). They should act as  $\cdot\text{OH}$  scavengers, since in our assay conditions we can rule out iron chelation and, thus, prevention of the Fenton reaction, as instead demonstrated using other models of oxidative stress [26]. Relating structure of the 2-benzazepine nitrones examined with their activity against  $\cdot\text{OH}$  production highlighted a 2-to-4-fold increase effect of the *gem*-dimethyl group at C-5 (**4**, **6** and **11**), compared with the respective mono-methyl derivatives (**3**, **5** and **10**). Such a higher activity of 5,5-dimethyl-substituted 2-benzazepine

nitrones could most likely be attributed to their structural peculiarity, inasmuch the presence of the 5-*gem*-dimethyl group should flatten the azepine ring, thus enhancing reactivity (radical attack onto nitrone fragment taking place from both sides of the azepine *pseudo* plane) and/or increasing stability of the resulting spin adducts [33]. The <sup>1</sup>H NMR data of the 5-*gem*-dimethyl derivatives, in agreement with geometry calculations on optimized structures, are reasonably consistent with this assumption. Indeed, the 4-CH<sub>2</sub> protons, which lose their axial and equatorial characters, resonate as singlet, showing their equivalence in the 5-*gem*-dimethyl-bearing derivatives **4**, **6** and **11**. In contrast, in 5-methyl derivatives **3**, **5** and **10** the C-4 protons' signals appear as two distinct doublets of doublets, with characteristic coupling constants for the coupling with the vicinal H-5, indicating that, in solution, the azepine ring preferably adopts a chair conformation with the methyl group at C-5 disposed equatorially [35]. As an additional SAR information, a methyl group to the nitrone C-1 (**5**, **6** and **12**), and to a lesser extent a phenyl group (**7**), diminish the <sup>•</sup>OH-scavenging activity of the nitrone group, because of its limited steric accessibility.

Mitochondria-specific effects, potentially contributing to neuroprotective properties, were determined as the ability of the examined 2-benzazepine nitrones to inhibit LPO and PCO (Table 2), induced in rat brain mitochondria by autoxidation of 6-OHDA. Our data showed that the investigated cyclic nitrones are one-two orders of magnitude more potent than PBN in protecting rat brain mitochondria against the oxidative insults, as measured by inhibition of TBARS formation (LPO) and protein carbonylation (PCO) increase. The IC<sub>50</sub> values determined in both assays are somehow correlated with the ability of the 2-benzazepine nitrones to scavenge <sup>•</sup>OH radicals (eqs. 1 and 2), suggesting that their neuroprotective activity may stem primarily, but not uniquely, from trapping these damaging radicals. Formation of nitrosyl radical species, which act as chain-breaking antioxidants [55], preventing propagation

of both LPO and PCO, may be supposed as an additional mechanism accounting for the observed protective properties.

From an SAR point of view, R<sup>1</sup> substitution with a methyl group appears as unfavorable, whereas the presence of the 5-*gem*-dimethyl group, more than the 5-methyl one, as well as the replacement of the 3-*gem*-dimethyl group with a spirocyclopentyl or spirocyclohexyl moiety, increases the inhibition potency against both LPO and PCO. A statistically significant equation (eq. 2) was derived, that correlates the in vitro LPO (but not PCO) inhibition data with <sup>•</sup>OH scavenging reactivity and lipophilicity of the 2-benzazepine nitrones, as described by the experimental log *k*'<sub>w</sub> parameter (but not by the calculated log *P*). According to other reported quantitative SARs [31], eq. 2 indicates that, their <sup>•</sup>OH scavenging potency being equal, 2-benzazepine nitrones having higher affinity for and greater residence time in the lipid phase are more effective in protecting the lipids from peroxidation. Among the most potent nitrones examined so far, compound **11** is noteworthy and is expected to be suitable for replacing PBN, whose oxygen-centered radical spin adducts are very short-lived [56], in the investigation of radicals in lipid membranes.

Investigating some systematic variations in the structure of 3,3-dimethyl-4,5-dihydro-3*H*-2-benzazepine *N*-oxide, some new cyclic nitrones have been singled out for their promising activity in protecting rat brain mitochondria from oxidative injury triggered by the PD-inducing neurotoxin 6-OHDA. Some of the examined derivatives, especially compound **11**, which incorporates into its structure the best features identified in this study (5-*gem*-dimethyl group and spiro C-3 cyclohexyl groups, and optimal lipophilicity), proved to be orders of magnitude more effective than PBN in vitro against 6-OHDA-induced mitochondrial lipid peroxide formation and protein oxidative damage.

The SAR information and lipophilicity data reported herein are important for drug design of potential therapeutics useful for treatment of neurodegenerative conditions in which

oxidative stress plays a role. Nevertheless, other properties must be taken into account for improving their therapeutic index. Indeed, while lipophilicity is generally recognized as a major property modulating antioxidant activity of cyclic nitrones in both LPO assay and maintenance of cell viability in models of oxidative injury in vitro [26,57], brain penetration and toxicity to neurons (ranging from sedation at moderate doses to death at very high doses), which in turn did not appear to linearly depend on lipophilicity and nitron brain level following ip injection in rats, respectively, strongly contribute to the therapeutic utility of cyclic nitrones in neurodegenerative disorders [57]. Results from our current investigation on selected 2-benzazepine nitrones in cell cultures and in vivo models of oxidative stress are expected to shed light on physicochemical properties of nitron derivatives which could readily penetrate the brain while retaining a desirable side effect profile.

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Table 1 – IC<sub>50</sub> values for inhibition of <sup>•</sup>OH production during 6-OHDA autoxidation

Compound	IC <sub>50</sub> (μM)
<b>PBN</b>	782 ± 39
<b>3</b>	48 ± 3.9*
<b>4</b>	17 ± 2.7*,§
<b>5</b>	235 ± 21*,§
<b>6</b>	63 ± 5.0*
<b>7</b>	46 ± 5.0*
<b>8</b>	57 ± 4.2*
<b>9</b>	7 ± 1.2*,§
<b>10</b>	37 ± 2.2*
<b>11</b>	19 ± 2.6*,§
<b>12</b>	278 ± 14*,§

The <sup>•</sup>OH formation was monitored by fluorimetry using terephthalic acid (THA) as a chemical dosimeter. The IC<sub>50</sub> values, determined using the 10-min time point, represent the concentrations of the test nitrones required to inhibit <sup>•</sup>OH formation by 50%. Values are means ± SEM from four independent determinations. Statistical significance at *P* < 0.05 (one-way ANOVA and Bonferroni test) was established in comparison with the corresponding control: \*, PBN; §, compd **3**.

Table 2 – Effects of 2-benzazepine nitrones on 6-OHDA-induced oxidative stress in brain mitochondria

Compound	IC <sub>50</sub> (μM)		Lipophilicity	
	LPO (TBARS)	PCOs	clog <i>P</i> <sup>a</sup>	log <i>k'</i> <sub>w</sub> <sup>b</sup>
<b>PBN</b>	8,500 ± 400	11,300 ± 182	1.25	
<b>3</b>	374 ± 27*	97 ± 9.4*	2.30	2.41
<b>4</b>	176 ± 11*,\$	40 ± 3.4*,\$	2.84	2.72
<b>5</b>	c	c	3.88	3.38
<b>6</b>	310 ± 11*	349 ± 14*,\$	4.42	3.37
<b>7</b>	137 ± 16*,\$	70 ± 7.8*	3.97	3.43
<b>8</b>	160 ± 8.1*,\$	144 ± 9.3*,\$	3.37	3.51
<b>9</b>	36 ± 6.2*,\$	53 ± 3.9*,\$	2.78	3.05
<b>10</b>	43 ± 2.9*,\$	91 ± 5.7*	3.34	3.55
<b>11</b>	20 ± 3.5*,\$	48 ± 4.0*,\$	3.88	3.47
<b>12</b>	c	184 ± 14*,\$	4.92	3.41

In vitro protective effects of nitrones against 6-OHDA autoxidation-dependent lipid peroxidation (LPO), as assessed by TBARS assay, and protein oxidative damage, as assessed by measuring the content of protein carbonyls (PCOs), in rat brain mitochondrial preparations. IC<sub>50</sub> values, determined using the 20-min time point, are means ± SEM from four independent determinations. Statistical significance at *P* < 0.05 (one-way ANOVA and Bonferroni test) was established in comparison with the corresponding control: \*, PBN; \$, compd **3**.

<sup>a</sup> Log of *n*-octanol-water partition coefficient calculated with the ACDLabs software, release 9.0 (Advanced Chemistry Development, Inc., Toronto, Canada).

<sup>b</sup> Log of RP-HPLC polycratic capacity factor, i.e., capacity factor extrapolated at 100% aqueous mobile phase.

<sup>c</sup> No statistically significant inhibition activity was attained up to the maximum tested concentration (1 mM).

## Figure Captions

**Fig. 1** – Structures of PBN spin trap and structurally related cyclic nitrones. The structures of 3,3-dimethyl-3,4-dihydroisoquinoline 2-oxides (**1**), 3,3-dimethyl-4,5-dihydro-3*H*-2-benzazepine 2-oxides (**2**), and their spiro C-3 cycloalkyl analogs, are shown, along with benzene ring X-substitution which proved to achieve significant improvements in activity over the parent compounds (X = H).

**Fig. 2** – Scheme of synthesis and structures of the examined 2-benzazepine nitrones.

**Fig. 3** – Representative recording of  $\cdot\text{OH}$  formation during the autoxidation of 6-OHDA in the absence (control) and presence of PBN and two 2-benzazepine nitrones (**3** and **9**). Incubations were performed in a 25 mM phosphate buffer (pH 7.5) at 37 °C. The concentrations used were: 10  $\mu\text{M}$  for 6-OHDA and 50  $\mu\text{M}$  for the nitrones. The  $\cdot\text{OH}$  formation is monitored by the fluorescence detected using terephthalic acid as a chemical dosimeter.

**Fig. 4** – The effects of 2-benzazepine nitrones **4**, **6**, **11** and **12** on the LPO induced by 6-OHDA (10  $\mu\text{M}$ ) autoxidation in rat brain mitochondria. LPO was assessed by monitoring the TBARS formation after incubation of mitochondrial preparations (1 mg/ml) at 37 °C for 20 min. Data are expressed as the means  $\pm$  SEM (n = 4).

**Fig. 5** – (A) Comparison between  $\log(1/\text{IC}_{50})$  values measuring the ability of the assayed 2-benzazepine nitrones to scavenge 6-OHDA-induced  $\cdot\text{OH}$  radicals ( $\text{pIC}_{50}^{\text{OH}}$ ) and inhibit lipid peroxidation ( $\text{pIC}_{50}^{\text{LPO}}$ ) in rat brain mitochondria. (B) Plot of measured (obs) vs calculated (calc), using equation 3,  $\text{pIC}_{50}^{\text{LPO}}$  values.