Heteroarylnitrones as Drugs for Neurodegenerative Diseases: Synthesis, Neuroprotective Properties, and Free Radical Scavenger Properties

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New 1,2,4-thiadiazolylnitrones and furoxanylnitrones were developed and evaluated as neuroprotective agents on a human neuroblastoma (SH-SY5Y) cells model. They inhibited at low micromolar concentrations the oxidative damage and the death induced by exposure to hydrogen peroxide. These heteroarylnitrones showed excellent peroxyl free radical absorbance capacities, analyzed by oxygen radical absorbance capacity (ORAC) assay with fluorescein as the fluorescent probe, ranging from 1.5- to 16.5-fold the value of the reference nitrone, α-phenyl-*N-tert*-butylnitrone (PBN). The electron spin resonance spectroscopy (ESR) demonstrated the ability of these derivatives to directly trap and stabilize oxygen, carbon, and sulfur-centered free radicals. These results demonstrated the potential use of these heteroarylnitrones as neuroprotective agents in preventing the death of cells exposed to enhanced oxidative stress and damage.

Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by oxidative metabolism are capable of damaging cellular components through molecular modifications to a polyunsaturated membrane's lipids, proteins, and nucleic acids.[1,2] Much evidence suggests that biological oxidation in the human body generates highly pathogenic ROS and RNS such as hydroxyl free radical ($^\bullet$OH), superoxide anion ($O_2^{\bullet-}$), peroxynitrite ($ONOO^-$), and lipid peroxide free radicals ($ROO^\bullet$), causing cellular injury.[3–5] These pathological events have important roles in many degenerative disorders, for example, atherosclerosis, rheumatoid arthritis, and several neurodegenerative diseases such as ischemic conditions, stroke, Parkinson's disease, and Alzheimer's disease.[6–8] The central nervous system (CNS) is especially sensitive to oxidative damage for reasons such as (a) high use of oxygen during the metabolic process and therefore greater production of ROS, (b) fewer antioxidants defenses than other organs, for example, liver and heart, (c) enrichment in the more easily peroxidable fatty acids, (d) areas with high levels of the Fe^{3+}/ascorbate pro-oxidant system that reduce H_2O_2 to form $^\bullet$OH, a potent oxidant.[9] Recently, it has been reported that β-amyloid solutions liberate hydrogen peroxide and, subsequently, $^\bullet$OH converted via Fenton's reaction.[10] Consequently, the search of effective treatments that prevent oxidative stress associated with premature aging and
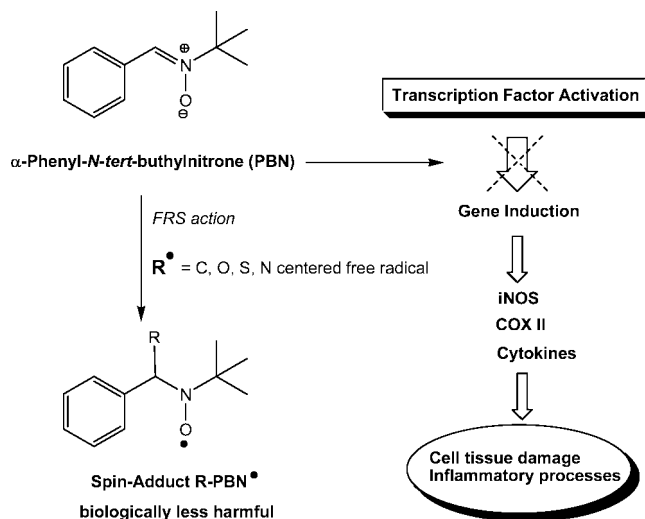

Figure 1. Antioxidant and neuroprotective mechanisms of action postulated for PBN.

neurodegenerative diseases is an important area of neurochemical research.

Recently, it has been proved that nitrone-free radical trap, i.e., α-phenyl-*N-tert*-butylnitrone (PBN,[a] Figure 1), could be used in the treatment of neurodegenerative diseases as well as in the prolongation of life span.[11] Initially, the neuroprotective activity was attributed to the nitrone group because of its ability to act as free radical scavenger (FRS, Figure 1). Subsequently, observations that PBN has a good neuroprotective activity at lower doses than those necessary to act as an effective FRS demonstrated the ability of PBN to inhibit signal transduction processes such as suppression of proinflammatory cytokines, genes, and mediators associated with enhanced neuroinflammatory processes (Figure 1).[12] The neuroprotective activity of the nitrone pharmacophore depends in great part on the connectivity and the nature of substituents on the nitrone group. In this sense, the chemical and pharmacological aspects of different heteroarylnitrones have been reviewed,[13] describing

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I, R₁= -C(CH₃)₃ R₂= -SO₃⁻Na⁺

II, R₁= -C₆H₁₁ R₂= -SO₃⁻Na⁺

III, R₁= -C(CH₃)₃ R₂= -SO₂N(CH₃)₂

Furyl Nitrones

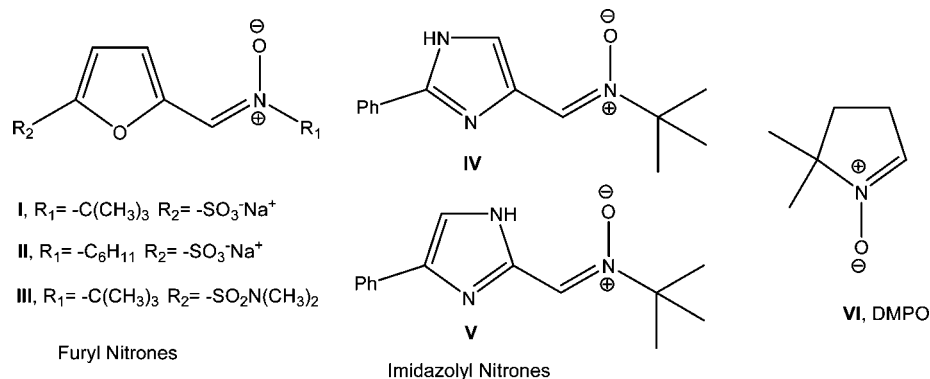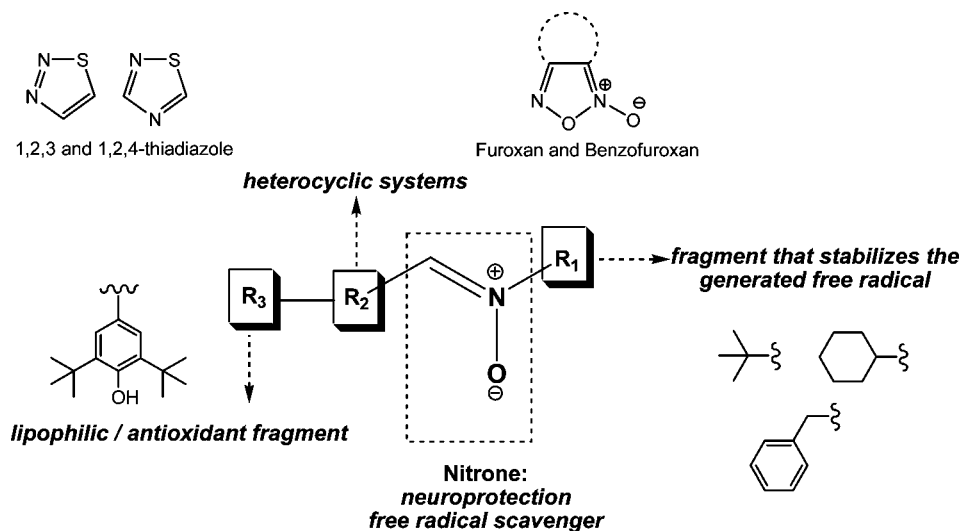Imidazolyl Nitrones

VI, DMPO

Figure 2. Heteroarylnitrones with neuroprotective and spin trapping properties.

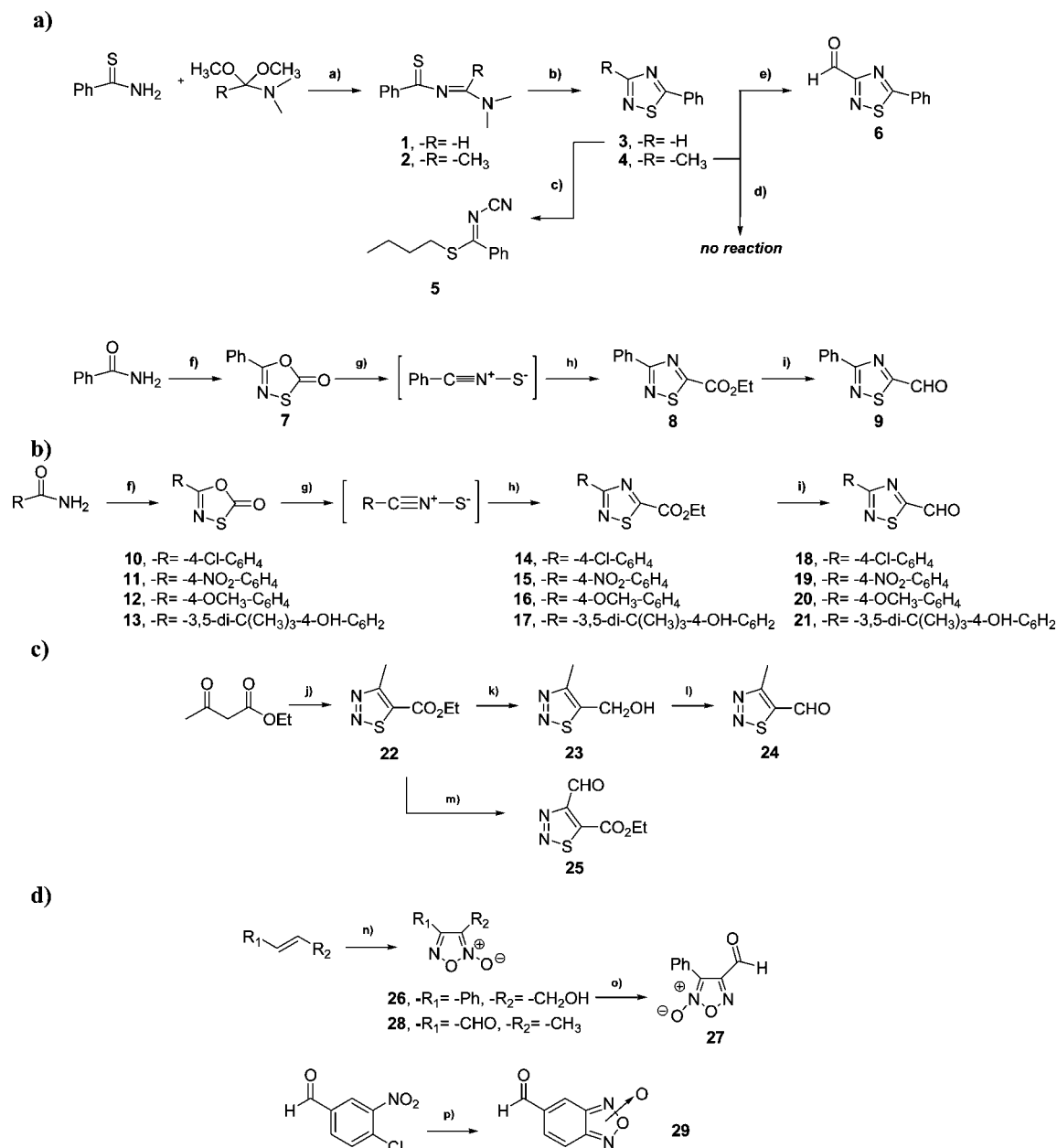**Scheme 1.** Heteroarylnitrones Designed as Potential Drugs for Neurodegenerative Diseases

1,2,3 and 1,2,4-thiadiazole

Furoxan and Benzofuroxan

heterocyclic systems

fragment that stabilizes the generated free radical

lipophilic / antioxidant fragment

Nitrone: *neuroprotection free radical scavenger*

some patented nitrone containing furans (**I**−**III**, Figure 2) with good activity against the neuronal cell damage induced by β-amyloid.[14] On the other hand, imidazolylnitrones (**IV** and **V**, Figure 2) has been developed and biologically evaluated, showing in vivo neuroprotective properties.[15] Additionally several nitrones has been widely used as spin trap for the specific detection of transient radicals (e.g., •OH, O₂•⁻, ROO•) or relatively stable radicals (e.g., NO) that are undetectable under normal conditions,[16] in the electron spin resonance (ESR) spectroscopy. With this aim two nitrones have been commonly used in the spin trapping technique: the linear nitrone PBN (Figure 1) and the cyclic nitrone DMPO (5,5-dimethyl-1-pyrroline *N*-oxide **VI**, Figure 2).

On the basis of that, herein we describe the design and synthesis of new heteroarylnitrones combining, in their structures, fragments able to show neuroprotection properties, such as nitrone moiety, antioxidant fragments, and heterocyclic groups able to stabilize the generated free radical (Scheme 1). In order to assess the protective effects of heteroarylnitrones, the effects of H₂O₂-injured in neuronal cells (SH-SY5Y) and in vitro unspecific cytotoxicity against J774-mouse macrophages were evaluated. Additionally, the antioxidant activities and the spin trapping properties have been investigated by evaluating the direct reaction with oxygen-, carbon-, and sulfur-centered free radicals by antioxidant assays and ESR spectroscopy.

Results and Discussion

Chemistry. Four different families of heteroarylnitrones were selected to study the influence on the biological activity: 1,2,3-

thiadiazole, 1,2,4-thiadiazole, 1,2,5-oxadiazole *N*-oxide, and benzo[1,2-*c*]1,2,5-oxadiazole *N*-oxide derivatives.[17,18] The nitrones syntheses were planned through the condensation between the corresponding heteroaromatic aldehyde and N-substituted hydroxylamine,[19] our first goal being the preparation of the corresponding aldehyde derivatives. For the synthesis of 3- and 5-formyl-1,2,4-thiadiazole aldehydes, **6** and **9**, respectively, the synthetic routes shown in Scheme 2a were employed. First, the reaction of benzothioamide with *N,N*-dimethylalkanamide dimethyl acetals produced the corresponding *N'*-(thioaroyl)-*N,N*-dimethylamidines **1** and **2** in excellent yield.[20] These intermediates were converted by amination−cyclization into the 1,2,4-thiadiazole **3** and **4**. The 3-formyl-1,2,4-thiadiazole **6** was obtained through bromination−oxidation of thiadiazole **4**. Attempts to obtain aldehyde **6** using formylation reaction between 5-phenyl-1,2,4-thiadiazole **3** and *n*-BuLi in DMF were unsuccessful. In these conditions, the ring-opening product, **5**, was mainly generated. Oxidation of the methyl substituent in compound **4** using selenium dioxide was also explored in the preparation of aldehyde **6**; however, no reaction was observed. Second, nitrile sulfide, obtained in situ through thermolysis of the 1,3,4-oxathiazol-2-one **7** (Scheme 2a), was treated with ethyl cyanoformate, yielding ester **8** via a 1,3-dipolar cycloaddition process.[21] Subsequently, the desired aldehyde **9** was prepared by reduction of **8** with DIBAL-H and subsequent oxidation with MnO₂. In order to know the 1,2,4-thiadiazole-substituents stereoelectronic/antioxidant effects (Scheme 1), different 3-aryl substitutions were studied (Scheme 2b). Consequently, aldehydes **18**, **20**, and **21** were prepared following the same synthetic

Scheme 2. Synthesis of Formyl-Heterocycle Derivatives[a]

a)

b)

c)

d)

[a] Reagents and conditions: (a) room temp; (b) HSA, Py, EtOH, room temp; (c) *n*-BuLi, THF, −78 °C, then DMF, 0 °C; (d) SeO$_2$, dioxane or ClCH$_2$CH$_2$Cl, reflux; (e) NBS, PDBO, CCl$_4$, reflux, then DSMO, 105 °C; (f) Cl(C=O)SCl, toluene, reflux; (g) decaline, 140−160 °C; (h) EtO$_2$C−CN; (i) (1) DIBAL-H, THF, 0 °C; (2) MnO$_2$, CHCl$_3$, reflux; (j) (1) H$_2$NNHCO$_2$Met, *p*-TsOH, toluene, reflux; (2) SOCl$_2$, 60 °C; (k) NaBH$_4$, EtOH, room temp; (l) MnO$_2$, CHCl$_3$, reflux; (m) NBS, PDBO, CCl$_4$, reflux, then DSMO, 120 °C; (n) NaNO$_2$, AcOH, 0 °C; (o) MnO$_2$, CHCl$_3$, room temp; (p) (1) NaN$_3$, DMSO, 60−70 °C; (2) DMSO, 90−100 °C.

route for aldehyde **9** preparation. Attempts to obtain the desired 4-NO$_2$ analogue **19** were unsuccessful because of the low stability of ester **15** in the reduction conditions (i) (Scheme 2b). The 1,2,3-thiadiazole carbonylic reagents were prepared from ethyl acetoacetate following a condensation with methyl carbazate, forming the corresponding acylhydrazone, and subsequent cyclization via Hurd−Mori reaction in the presence of thionyl chloride (Scheme 2c).[22] Then thiadiazole **22** was transformed into the key aldehydes **24** and **25** by reduction/oxidation for the first case and by benzylic-like bromination/oxidation for the second. Finally, the synthetic procedures for furoxan and benzofuroxan systems included, for the first heterocycle, addition to alkenes followed by intramolecular cyclization and thermointramolecular cyclization for the second one (Scheme 2d). Consequently, the reaction of cinnamyl

alcohol or crotonaldehyde with NaNO$_2$ in AcOH produced the furoxan derivatives **26** and **28**, respectively.[23] Alcohol **26** was converted into the aldehyde **27** by oxidation with MnO$_2$.[23] The formylbenzofuroxan **29** was obtained from 4-chloro-3-nitrobenzaldehyde in a one-pot process via nucleophilic substitution with the azide anion and subsequent cyclization by pyrolysis of the corresponding *o*-nitrophenylazide intermediate.[24] The desired nitrones, **30−41**, were obtained in good yield for reaction between the corresponding aldehydes **6**, **9**, **18**, **20**, **21**, **24**, **25**, **27−29** and N-substituted hydroxylamine hydrochloride in the presence of NaHCO$_3$ (Scheme 3). Taking into account that the nitrone stability, as well as the stability of the corresponding spin-adduct (nitroxyl free radical), is highly influenced by the N-alkyl substituent,[13] we developed derivatives **31−33** by

Scheme 3. Synthesis and Structure of New Heteroarylnitrones[a]

Ref.	heterocycle-	-R²	-R¹
30	Ph–(1,2,4-thiadiazole, Me)	-	-*t*-butyl
31	R²–phenyl–(1,3,4-thiadiazole)	-H	-*t*-butyl
32		-H	-cyclohexyl
33		-H	-benzyl
34		-Cl	-*t*-butyl
35		-OCH₃	-*t*-butyl
36	HO–di-*t*-butylphenyl–(1,3,4-thiadiazole)	-	-*t*-butyl
37	Me–(1,2,3-thiadiazole)	-	-*t*-butyl
38	(1,2,3-thiadiazole)–CO₂Et	-	-*t*-butyl
39	Me,Ph–furoxan (*)	-	-*t*-butyl
40	Me–furoxan	-	-*t*-butyl
41	Me–benzofuroxan	-	-*t*-butyl

[a] Asterisk (∗) indicates at room temperature.

varying R¹ (Scheme 3) in order to evaluate the effect of this modulation in the bioactivity studies.

All the structures of the desired nitrones, **30**−**41**, were established by NMR (¹H, ¹³C, HMQC, and HMBC experiments), IR, and MS. The purity was established by TLC and microanalysis. The stereochemistry around the olefinic carbon−nitrogen bond of the nitrone function was established using NOE-diff experiments, and they are Z-isomers in all the cases.

Biology. Neuroprotective Activity. The neuroprotective activity of the new developed heteroarylnitrones, **30**−**41**, was studied using a human neuronal-like cellular system (SH-SY5Y cells) exposed to H_2O_2 as oxidative damaging agent.[25] First, SH-SY5Y cytotoxicity of the developed heteroarylnitrones and PBN was evaluated in vitro in the range 0.05−10 μM for 24 h. The cell viability was determined by measuring the activity of lactate dehydrogenase (LDH) released into the culture medium and expressed as the difference between nitrones-treated and untreated cells. All the nitrones displayed no cytotoxicity for neuronal cells, 100% of survival, at assayed neuroprotective doses (10 μM). Second, each one of the heteroarylnitrones, at 10 μM doses, was administered to the cells 1 h before the incubation with H_2O_2. The cell viability was determined by measuring the activity of LDH released and expressed as the difference between nitrones-treated and untreated cells. The basal percentage of viable neurons after treatment with 100 μM H_2O_2 was 45%, and *N*-acetylcysteine (NAC) was used as positive test at 5 mM (Figure 3). The values summarized in Table 1 show that the 1,2,4-thiadiazolylnitrones **30**−**36** and the furoxanylnitrone **40** protect similarly to or slightly higher against the cellular damage than the parent nitrone PBN, with values between 33% and 46%. Interestingly, the treatment of SH-SY5Y cells with the 1,2,4-thiadiazolylnitrone **36** at 1 μM significantly protects against H_2O_2 induced neurotoxicity, with a value of 20% (Figure 3). Furthermore, the benzofuroxanylnitrone **41** showed lower capacity to protect neuroblastoma human cells from death induced by oxidative stress than PBN, while the 1,2,3-thiadiazolylnitrones (**37** and **38**) and furoxanylnitrone **39** did not show neuroprotective effects. These results revealed the consequence of the connectivity and the nature of the nitrone-pharmacophore substituents in the neuroprotective activity. The 1,2,4-thiadiazolylnitrones **31**, **32**, and **33**, substituted in the nitrogen atom of the nitrone by a *tert*-butyl, cyclohexyl, and benzyl group, respectively, exhibited differential activity, indicating some *N*-alkyl moieties dependence.
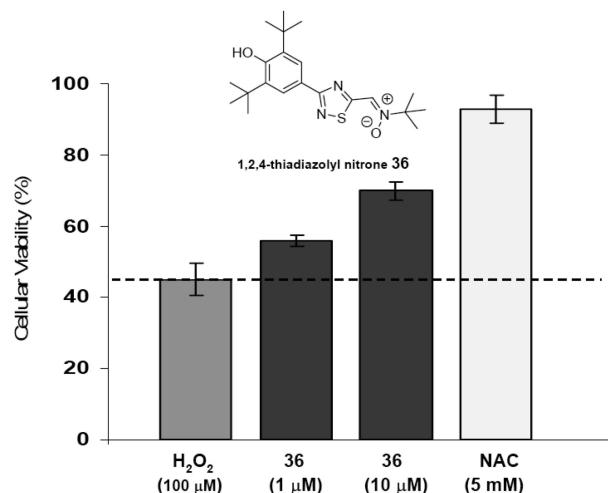
Figure 3. Cellular viability in different experimental conditions: H_2O_2 (100 μM), nitrone **36** (at 10 and 1 μM), and NAC (5 mM) used as positive test. Viability was determined through LDH activity after 24 h of incubation (mean ± SD values from three independent experiments).

Table 1. Effect of Heteroarylnitrones on Cell viability in SH-SY5Y Cells Preincubated with H_2O_2 (100 μM) and Heteroarylnitrones Cytotoxicities (J774 Mouse Macrophages)

ref	difference in cell viability[a−c]	% neuroprotection [c,d]	J774 IC$_{50}$ (μM)[e]
PBN	20.6 ± 0.2	37	>400
30	20.0 ± 1.9	36	>400
31	20.7 ± 9.4	38	>400
32	23.6 ± 10.7	43	100
33	18.1 ± 3.0	33	240
34	19.1 ± 2.1	34	200
35	25.0 ± 5.2	46	>400
36	25.2 ± 5.6	46	>400
37	0		>400
38	0		>400
39	0		100
40	23.0 ± 2.4	42	>400
41	13.4 ± 6.0	24	150

[a] The cell viability was measured with LDH activity being 100% the basal value. The basal value was obtained from the cells that did not receive any treatment. Difference of cell viability was determined by the difference between LDH activity in nitrones−H_2O_2-treated cells and LDH activity in H_2O_2-treated cells and normalized to 100%. [b] Data are expressed as the mean ± SD ($p < 0.001$). Experiments were done in triplicate. [c] Doses of heteroarylnitrones are 10 μM. [d] Basal percentage of viable neurons after treatment with 100 μM H_2O_2 is 45%. The percentages of neuroprotection are determined from the basal values. [e] IC$_{50}$: concentration that produces 50% inhibitory effect. The results are the mean values of two different experiments with SD values less than 10% in all cases.

Mammal Cytotoxicity. Unspecific mammalian cytotoxicity of the developed heteroarylnitrones was evaluated in vitro in the range 50−400 μM, using J774 mouse macrophages as the cellular model (Table 1).[26] In the study PBN was included as a neuroprotective reference compound. The developed heteroarylnitrones displayed no cytotoxicity for mammalian cells at its neuroprotective doses (10 μM). Furthermore, the most active heteroarylnitrones, **35**, **36** and **40**, show low mammalian cytotoxicity in the assayed in vitro model, these compounds being as cytotoxic as the parent compound PBN. Moreover, the IC$_{50}$ values summarized in Table 1 show that the 1,2,4-thiadiazolyl, **32−34**, furoxanyl, **39**, and benzofuroxanylnitrones, **41**, are a little more toxic against this cellular system than the parent compound PBN.

Blood−Brain Barrier Permeation Studies. For the 1,2,4-thiadiazolylnitrones **35** and **36**, with the best neuroprotective
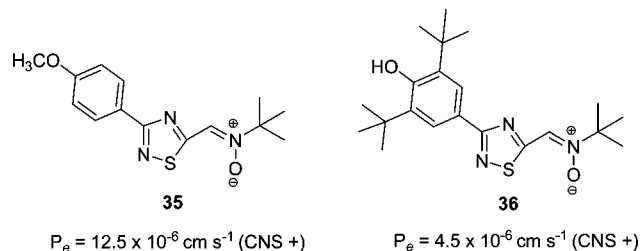

Figure 4. Prediction of the brain penetration using PAMPA-BBB assay by 1,2,4-thiadiazolylnitrones **35** and **36** (CNS +: compounds able to cross the BBB).

Table 2. Free-Radical Scavenger/Antioxidant Activity of Heteroarylnitrone Derivatives Using ORAC$_{FL}$ Method and DPPH Captured

ref	ORAC (PBN equiv)[a]	RP of DPPH (%)[b]
PBN	1.0	0 (20[c])
30	2.0 ± 0.1	41 ± 3
31	5.5 ± 0.1	0
32	4.6 ± 0.2	11 ± 3
33	3.5 ± 0.3	0
34	1.5 ± 0.2	0
35	7.0 ± 0.4	10 ± 4
36	6.0 ± 0.2	40 ± 3
37	2.5 ± 0.1	0
38	3.0 ± 0.1	0
39	6.0 ± 0.2	0
40	2.5 ± 0.1	8 ± 1
41	16.5 ± 0.4	17 ± 5

[a] Data are expressed as μmol of PBN equiv/μmol of tested heteroarylnitrones and are the mean ($n = 3$) ± SD. [b] All heteroarylnitrones were assayed at 0.1 mM. [c] Evaluated at 10 mM.

profile and the lowest mammalian cytotoxicity (Table 1), the capacity to cross the blood−brain barrier (BBB) by means of a parallel artificial membrane permeation assay for BBB (PAMPA-BBB) using a lipid extract of porcine brain was determined. The assay validation was made by comparing experimental permeabilities of 20 commercial drugs to reported values.[27] From this experiment and taking into account the limit established by Di et al. for BBB permeation, compounds with permeability (P_e) above 4.4×10^{-6} cm s^{-1} could cross the BBB (Figure 4).[28]

Free-Radical Scavenger Properties Antioxidant Activity. The antioxidant activity of the newly developed heteroarylnitrones was determined using two in vitro assays, the oxygen radical absorbance capacity (ORAC) and the measurement of reduction percentage (RP) of the stable diphenylpicrylhydrazyl free radical (DPPH). ORAC assay[29] measures peroxyl free radical scavenger compounds capability and using fluorescein (FL) as the fluorescent probe. The peroxidation of FL yields nonfluorescent products at 520 nm, so the remaining fluorescence of FL in the presence of the free radical scavenger compound is a measure of the antioxidant activity of the test compound. In this assay the results were expressed in reference to PBN, as PBN equiv (μmoles of PBN equivalents per μmoles of tested compound) (Table 2). On the other hand, the RP of DPPH is based on the reaction between the deep-violet stable free radical (characterized by an absorption band in ethanol solution centered at 520 nm) and the studied free radical scavenger measuring the absorbance of nitrone-treated and untreated DPPH solutions (Table 2).[30] The peroxyl free radical scavenger property of our nitrones, according to ORAC assay, was better than that of PBN, being 1.5−16.5 higher than PBN value. The benzofuroxanylnitrone **41** showed the highest ORAC$_{FL}$ value (16.5 PBN equiv), whereas the 1,2,4-thiadiazolylnitrone **34** showed the lowest ORAC$_{FL}$ value (1.5 PBN equiv). Similar results of antioxidant
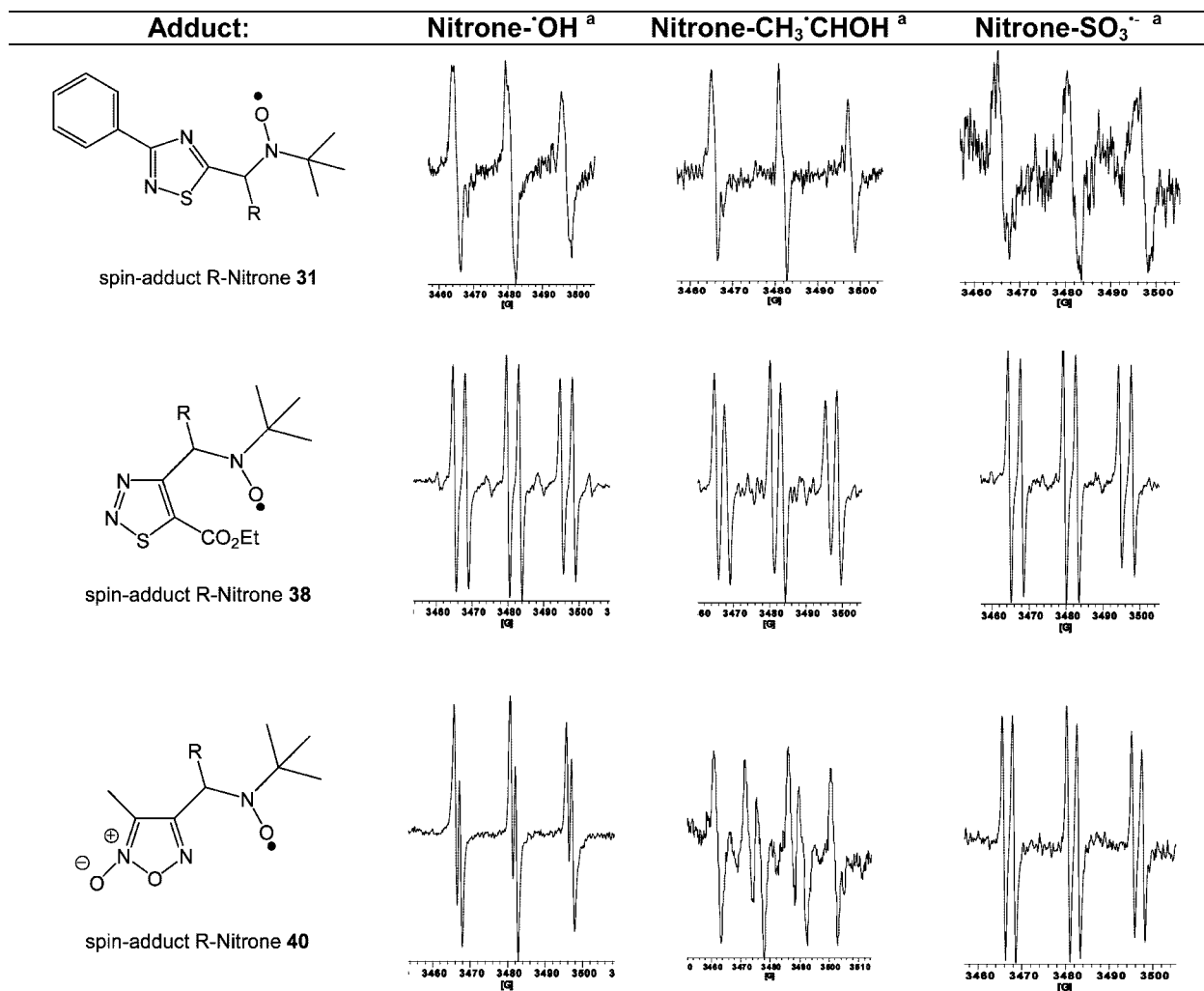
Figure 5. ESR spectra obtained in the trapping of $^\bullet$OH, $CH_3 {}^\bullet CH_2OH$, and $SO_3 {}^{\bullet-}$ free radicals with nitrones **31**, **38**, and **40**. Superscript "a" in the heading entries indicates that $^\bullet$OH, $CH_3 {}^\bullet CHOH$, and $SO_3 {}^{\bullet-}$ free radicals are generated by Fenton's reaction.

activity were obtained through DPPH assay. The heteroarylnitrones were tested at 0.1 mM, being more potent antioxidants than PBN, which at 10 mM possess 20% of DPPH reduction. In this sense, the 1,2,4-thiadiazolylnitrones **30** and **36** showed the highest antioxidant values, 41 and 40 DPPH RP, respectively.

Electron Spin Resonance Measurements. Initially the $^\bullet$OH-spin trapping ability of the developed heteroarylnitrones was investigated using ESR spectroscopy. The $^\bullet$OH free radical was generated in situ from a Fenton reaction system. All the assayed heteroarylnitrones, except the thiadiazolylnitrones **32** and **33** and furoxanylnitrone **39**, gave strong ESR signals (Figure 5), and the hyperfine splitting (HFS) constants for the different spin adducts are reported in Table 3. These data indicate that in general our heteroarylnitrones generate stable spin adducts with $^\bullet$OH, like previously reported imidazolylnitrones.[31] The absence of signal in the cases of nitrones **32** and **33** indicated that the cyclohexyl and benzyl groups affect the stability of the spin adduct generated under the studied conditions.

In order to study the capability of the developed nitrones as spin trapping against other free radical species, we selected one nitrone from each proposed family. Consequently, the 1,2,4-thiadiazolylnitrone **31**, the 1,2,3-thiadiazolylnitrone **38**, and the furoxanylnitrone **40** were studied as both ethanol and sulfite free radical spin trappings. The ESR signals obtained for these heteroarylnitrones are given in Figure 5. The ESR signals for the free radical adducts with nitrones **31**, **38**, and **40** indicated

Table 3. HFS Constants for the Heteroarylnitrone−$^\bullet$OH Spin Adduct

nitrone−$^\bullet$OH^{a,b} spin adduct, ref	a_N (G)^c	$a_{H\beta}$ (G)^c
30	14.8	2.4
31	15.7	0.9
32	ns^d	ns^d
33	ns^d	ns^d
34	15.3	0.6
35	15.7	0.7
36	14.0	2.6
37	14.5	2.6
38	15.5	4.0
39	ns^d	ns^d
40	15.8	2.1
41	14.1	2.2

^{a} $^\bullet$OH was generated from a standard Fenton system: $Fe(NH_4)_2 (SO_4)_2 \cdot 6H_2O$ (0.5 mM) was added to a solution of nitrone (50 mM) and H_2O_2 (0.5 mM) in phosphate buffer (0.1 M). ^{b} The ESR spectrum of the spin adduct was recorded 30 s after addition of $FeSO_4$. ^{c} G: gauss. ^{d} ns: no signals in the assayed conditions.

that these nitrones are also very efficient to trap and stabilize both carbon- and sulfur-centered free radicals, $CH_3 {}^\bullet CHOH$ and $SO_3 {}^{\bullet-}$, respectively. These results suggest that our nitrones have great capacity to scavenge free radicals and potential use to prevent or reduce the damage caused by radicals in several degenerative disorders. However, because nitrone **38** is very efficient in spin trapping (i.e., $^\bullet$OH, $CH_3 {}^\bullet CHOH$, $SO_3 {}^{\bullet-}$, Figure 5), it does not have neuroprotective effects in the neuroblastoma

human SH-SY5Y system (Table 1), showing that the neuroprotective activity might not exclusively reside in its capacity to scavenge free radicals. These results are similar to those recently reported for other heteroarylnitrones.[32]

Conclusion

New series of thiadiazolyl and furoxanylnitrones have been synthesized and biologically evaluated as potential neuroprotective agents for the treatment of neurodegenerative disorders. The designed heteroarylnitrones were efficiently obtained through the condensation between heteroaromatic aldehydes and N-monosubstituted hydroxylamines with good to excellent yields and chromatographically isolated as pure Z-isomers. They show excellent free radical scavenger capacity and good neuroprotective effects without cellular toxicity. The ESR spectroscopy demonstrated the ability of these heteroarylnitrones to directly scavenge different free radicals (i.e., $^•$OH, $CH_3^•$CHOH, $SO_3^{•-}$). Taking into account all the results, the designed heteroarylnitrones have demonstrated a therapeutic potential as neuroprotective agents in preventing the death of cells exposed to enhanced oxidative stress and damage. Further structural optimization, QSAR, and in vivo neuroprotective property studies are currently underway.

Experimental Section

Compounds **3**, **4**,[20] **8**,[21] **14**−**17**,[33−36] and **26**−**29**[23] were prepared according to literature procedures. Melting points were determined with an electrothermal melting point apparatus (Electrothermal 9100) and are uncorrected. Proton and carbon NMR spectra were recorded on a Bruker DPX-400 spectrometer. The chemical shifts values are expressed in ppm relative to tetramethylsilane as internal standard. Mass spectra were determined in a MSD 5973 Hewlett-Packard or LC/MSD series 100 Hewlett-Packard spectrometer using electronic impact (EI) or electrospray ionization (ESI), respectively. Microanalyses were performed in a Fisons EA 1108 CHNS-O equipment and were within ±0.4% of the calculated compositions. Column chromatography was carried out using Merck silica gel (60−230 mesh). Most chemicals and solvents were analytical grade and used without further purification. All the reactions were carried out in a nitrogen atmosphere. The typical workup included washing with brine and drying the organic layer with sodium sulfate before concentration.

3-Formyl-5-phenyl-1,2,4-thiadiazole (6). A mixture of **4**[20] (0.4 g, 2.3 mmol), N-bromosuccinimide (0.45 g, 2.5 mmol), and dibenzoyl peroxide (0.08 g, 0.34 mmol) in dry CCl_4 (8.0 mL) was heated at reflux for 16 h. Then the mixture was allowed to cool to room temperature, the succinimide was collected and washed with CCl_4 (10.0 mL), and the organic layer was evaporated in vacuo. 3-Bromomethyl-5-phenyl-1,2,4-thiadiazole was obtained as an oil (~40% by ^{1}H NMR signals) and was used in the next reaction without further purification. ^{1}H NMR (CDCl$_3$, 400 MHz) δ: 4.69 (s, 2H), 7.45−7.55 (m, 3H), 7.90−8.00 (m, 2H). The crude of the bromination process in DMSO (6.0 mL) was stirred and warmed at 105 °C during 1.5 h. Subsequently, the mixture was poured into saturated sodium chloride solution (30.0 mL) and extracted with EtOAc (3 × 20.0 mL). After the workup of the combined organic layers, the residue was purified by column chromatography (SiO$_2$, petroleum ether/EtOAc (9:1)), yielding derivative **6** as a yellow oil (130 mg, 30% two steps). ^{1}H NMR (CDCl$_3$) δ: 7.45−7.65 (m, 3H), 7.90−8.10 (m, 2H), 10.30 (s, 1H). EI-MS, m/z (abundance, %): 190 (M$^{+•}$, 27), 135 (100), 103 (15).

General Procedure for the Synthesis of 3-Aryl-5-formyl-1,2,4-thiadiazole Derivatives (9 and 18−21). A solution of the corresponding ester[20,33−36] (1 equiv) in THF (4 mL/mmol) as solvent was cooled at 0 °C. Then a solution of DIBAL-H (1 M in hexane, 2−3 equiv) was added slowly and the final mixture was stirred during 3 h. The reaction mixture is diluted with EtOAc and washed with HCl (1 M). After the workup, the residue, corresponding to

the 3-aryl-5-hydroxymethyl-1,2,4-thiadiazole (pure by TLC), was used in the next reaction without further purification. Subsequently, a mixture of the corresponding alcohol (1 equiv) and MnO$_2$ (10 equiv) in CHCl$_3$ (12 mL/mmol) as solvent was heated at reflux until absence of the alcohol (checked by TLC). The resulting dispersion was then filtered through a short pad of Celite, the organic phase concentrated in vacuo and the residue purified by column chromatography (SiO$_2$, petroleum ether/EtOAc (8:2)) to afford spectroscopically pure compounds.

5-Hydroxymethyl-3-phenyl-1,2,4-thiadiazole. ^{1}H NMR (CDCl$_3$) δ: 2.83 (t, 1H), 5.17 (d, 2H, J = 5.5 Hz), 7.45−7.51 (m, 3H), 8.24−8.30 (m, 2H). EI-MS, m/z (abundance, %): 192 (M$^{+•}$, 38), 135 (100), 103 (24), 77 (20).

5-Formyl-3-phenyl-1,2,4-thiadiazole (9). Yellow solid, 0.13 g (25%, two steps). ^{1}H NMR (CDCl$_3$) δ: 7.52 (m, 3H), 8.33 (m, 2H), 10.19 (s, 1H). ^{13}C NMR (CDCl$_3$) δ: 184.3 (C5), 182.5 (CHO), 175.1 (C3), 131.9 (Cq), 131.0 (Co), 128.9 (Cm), 128.3 (Cp). EI-MS, m/z (abundance, %): 190 (M$^{+•}$, 84), 135 (100), 103 (30), 77 (24).

3-(4-Chlorophenyl)-5-hydroxymethyl-1,2,4-thiadiazole. ^{1}H NMR (CDCl$_3$) δ: 2.70 (s, 1H), 5.05 (s, 2H), 7.38 (d, 2H, J = 8.5 Hz), 8.15 (d, 2H, J = 8.5 Hz). EI-MS, m/z (abundance, %): 226 (M$^{+•}$, 51), 169 (100), 137 (35), 102 (15).

3-(4-Chlorophenyl)-5-formyl-1,2,4-thiadiazole (18). Yellow solid, 0.28 g (44%, two steps). ^{1}H NMR (DMSO-d$_6$) δ: 7.67 (d, 2H, J = 8.5 Hz), 8.28 (d, 2H, J = 8.7 Hz), 10.16 (s, 1H). ^{13}C NMR (DMSO-d$_6$) δ: 180.2 (CHO), 179.1 (C5), 173.52 (C3), 137.6 (Cq), 132.7 (Cq), 130.6 (Co), 130.0 (Cm). EI-MS, m/z (abundance, %): 224 (M$^{+•}$, 75), 169 (100), 137 (39), 102 (18).

5-Hydroxymethyl-3-(4-methoxyphenyl)-1,2,4-thiadiazole. ^{1}H NMR (CDCl$_3$) δ: 2.75 (s, 1H), 3.87 (s, 3H), 5.15 (s, 2H), 7.01 (d, 2H, J = 8.6 Hz), 8.42 (d, 2H, J = 8.6 Hz). EI-MS, m/z (abundance, %): 222 (M$^{+•}$, 49), 167 (100), 135 (57).

5-Formyl-3-(4-methoxyphenyl)-1,2,4-thiadiazole (20). Yellow solid, 0.13 g (31%, two steps). ^{1}H NMR (CDCl$_3$) δ: 3.85 (s, 3H), 7.04 (d, 2H, J = 8.6 Hz), 8.31 (d, 2H, J = 8.7 Hz), 10.19 (s, 1H). ^{13}C NMR (CDCl$_3$) δ: 181.2 (CHO), 178.3 (C5), 172.3 (C3), 160.9 (Cp), 130.1 (Co), 114.5 (Cm), 54.9 (OCH$_3$). EI-MS, m/z (abundance, %): 220 (M$^{+•}$, 99), 165 (100), 150 (45), 133 (75).

3-(3,5-Di-*tert*-butyl-4-hydroxyphenyl)-5-hydroxymethyl-1,2,4-thiadiazole. ^{1}H NMR (CDCl$_3$) δ: 1.52 (s, 18H), 2.93 (s, 1H), 5.16 (s, 2H), 5.53 (s, 1H), 8.14 (s, 2H). ESI-MS, m/z: 321 (M$^{+•}$ + H).

3-(3,5-Di-*tert*-butyl-4-hydroxyphenyl)-5-formyl-1,2,4-thiadiazole (21). Brown solid, 0.14 g (27%, two steps). ^{1}H NMR (CDCl$_3$) δ: 1.54 (s, 18H), 5.61 (s, 1H), 8.21 (s, 2H), 10.23 (s, 1H). ^{13}C NMR (CDCl$_3$) δ: 183.9 (C5), 182.8 (CHO), 175.9 (C3), 156.6 (Cp), 136.4 (Cm), 125.7 (Co), 125.3 (Cq), 34.4 (C(CH$_3$)$_3$), 30.2 (C(CH$_3$)$_3$). ESI-MS, m/z: 319 (M$^{+•}$ + H).

5-Ethoxycarbonyl-4-methyl-1,2,3-thiadiazole (22). A mixture of ethyl acetoacetate (4.9 mL, 38.5 mmol), methyl carbazate (3.8 g, 42.4 mmol), and p-TsOH (catalytic amounts) in toluene (120.0 mL) was heated at reflux for 2 h. Then the solvent was concentrated in vacuo and the crude of the reaction was used in the next reaction without further purification. The acylhydrazone obtained was cooled at 0 °C, and SOCl$_2$ (20.0 mL, 270 mmol) was added dropwise with stirred during 10 min. The final mixture was stirred during 2 h at 60 °C, the excess of SOCl$_2$ was evaporated in vacuo, and the residue was neutralized with aqueous saturated NaHCO$_3$ and extracted with EtOAc (3 × 10 mL). After the workup the organic solvent was evaporated in vacuo and the crude purified by column chromatography (SiO$_2$, petroleum ether/EtOAc (8:2)), yellow oil, 1.63 g (25%). ^{1}H NMR (CDCl$_3$) δ: 1.55 (t, 3H), 2.95 (s, 3H), 4.42 (q, 2H). ^{13}C NMR (CDCl$_3$) δ: 162.5 (C4), 160.1 (C=O), 139.8 (C5), 62.8 (CH$_2$CH$_3$), 14.5 (CH$_2$CH$_3$ + CH$_3$). ESI-MS, m/z: 173.0 (M$^{+•}$ + H).

5-Hydroxymethyl-4-methyl-1,2,3-thiadiazole (23). A solution of **22** (0.8 g, 4.7 mmol) in EtOH (8.0 mL) was cooled at 0 °C. Then NaBH$_4$ (0.3 g, 7.9 mmol) was added slowly and the final mixture was stirred during 2 h at room temperature. The organic solvent was evaporated in vacuo, and the residue was treated with H$_2$O (50.0 mL) and extracted with EtOAc (3 × 10.0 mL). After the

workup the organic solvent was evaporated in vacuo. The residue was purified by column chromatography (SiO$_2$, petroleum ether/ EtOAc (7:3)), yielding derivative **23** as a yellow oil (0.42 g, 70%). ^{1}H NMR (CDCl$_3$) δ: 2.55 (s, 3H), 4.55 (bs, 1H), 4.94 (s, 2H). ^{13}C NMR (CDCl$_3$) δ: 154.4 (C5), 152.9 (C4), 56.3 (CH$_2$OH), 12.1 (CH$_3$). ESI-MS, *m/z*: 131 (M$^{+\bullet}$ + H).

5-Formyl-4-methyl-1,2,3-thiadiazole (24). A mixture of **23** (0.49 g, 3.77 mmol) and MnO$_2$ (3.28 g, 37.7 mmol) in CHCl$_3$ (45 mL) as solvent is heated at reflux until absence of the alcohol **23** (checked by TLC). The resulting dispersion was then filtered through a short pad of Celite, the organic phase concentrated in vacuo, and the residue purified by column chromatography (SiO$_2$, petroleum ether/EtOAc (8:2)), yielding derivative **24** as a yellow oil (105 mg, 26%). ^{1}H NMR (CDCl$_3$) δ: 3.05 (s, 3H), 10.26 (s, 1H). ^{13}C NMR (CDCl$_3$) δ: 184.3 (CHO), 156.2 (C5), 154.2 (C4), 14.4 (CH$_3$). ESI-MS, *m/z*: 129 (M$^{+\bullet}$ + H).

5-Ethoxycarbonyl-4-formyl-1,2,3-thiadiazole (25). A mixture of **22** (1.0 g, 5.8 mmol), *N*-bromosuccinimide (1.14 g, 6.4 mmol), and dibenzoyl peroxide (0.06 g, 0.23 mmol) in dry CCl$_4$ (40.0 mL) was heated at reflux for 16 h. Then the mixture was allowed to cool to room temperature, the succinimide was collected and washed with CCl$_4$, and the organic layer was evaporated in vacuo. DMSO (10.0 mL) was added, and the mixture was warmed at 120 °C during 3 h. Then EtOAc (50 mL) was added and the organic layer was washed with saturated sodium chloride solution (40.0 mL). After the workup the organic solvent was evaporated in vacuo and the residue purified by column chromatography (SiO$_2$, petroleum ether/ EtOAc (8:2)), yielding a yellow oil that crystallized at 4 °C (0.4 g, 40%). ^{1}H NMR (CDCl$_3$) δ: 1.48 (t, 3H), 4.56 (q, 2H), 10.67 (s, 1H). ^{13}C NMR (CDCl$_3$) δ: 186.1 (CHO), 157.2 (C4), 161.8 (C=O), 141.5 (C5), 64.2 (*C*H$_2$CH$_3$), 14.1 (CH$_2$*C*H$_3$). ESI-MS, *m/z*: 187 (M$^{+\bullet}$ + H).

General Procedure for the Synthesis of α-Heteroaryl-*N*-alkyl- nitrone Derivatives (30−41). A mixture of the corresponding aldehyde (1 equiv), *N*-alkylhydroxylamine hydrochloride (1.5 equiv), and sodium bicarbonate (1.5 equiv) in absolute ethanol (6 mL/mmol) as solvent was heated at 60 °C until the carbonyl compound was not present (checked by TLC). The solvent was removed in vacuo and the reaction mixture diluted with H$_2$O and extracted with EtOAc. After the workup of the combined organic layers, the residue was purified by column chromatography (SiO$_2$, mixtures of petroleum ether/EtOAc).

α(Z)-(5-Phenyl-1,2,4-thiadiazol-3-yl)-*N-tert*-butylnitrone (30). Yel- low solid (87%); mp 111.0−113.0 °C; ^{1}H NMR (CDCl$_3$) δ: 1.63 (s, 9H), 7.48−7.56 (m, 3H), 7.97 (d, 2H), 8.18 (s, 1H). ^{13}C NMR (CDCl$_3$) δ: 187.4 (C5), 166.5 (C3), 132.1 (Cp), 129.3 (Cm), 127.5 (Co), 123.2 (CH=N), 73.0 (*C*(CH$_3$)$_3$), 28.3 (C(*C*H$_3$)$_3$). ESI-MS, *m/z*: 262 (M$^{+\bullet}$ + H). IR (KBr) ν: 1707, 1561, 1469, 1435, 1248, 1152, 773, 669, 695. Anal. Calcd for (C$_{13}$H$_{15}$N$_3$OS): C, 59.74; H, 5.79; N, 16.08; S, 12.27. Found: C, 59.46; H, 5.86; N, 15.97; S, 12.27.

α(Z)-(3-Phenyl-1,2,4-thiadiazol-5-yl)-*N-tert*-butylnitrone (31). Yel- low solid (82%); mp 107.0−108.0 °C. ^{1}H NMR (CDCl$_3$) δ: 1.66 (s, 9H), 7.44−7.50 (m, 3H), 8.29−8.32 (m, 2H), 8.63 (s, 1H). ^{13}C NMR (CDCl$_3$) δ: 175.8 (C5), 172.0 (C3), 133.2 (Cq), 130 (Co), 128.9 (Cm), 128.2 (Cp), 126.1 (CH=N), 71.9 (*C*(CH$_3$)$_3$), 28.3 (C(*C*H$_3$)$_3$). EI-MS, *m/z* (abundance, %): 261 (M$^{+\bullet}$, 16), 205 (42), 135 (19), 103 (19), 57 (100). IR (KBr) ν: 1544, 1470, 1414, 1344, 1234, 1112, 913, 716, 696, 558. Anal. Calcd for (C$_{13}$H$_{15}$N$_3$OS): C, 59.74; H, 5.79; N, 16.08; S, 12.27. Found: C, 59.35; H, 5.74; N, 15.89; S, 12.08.

α(Z)-(3-Phenyl-1,2,4-thiadiazol-5-yl)-*N*-cyclohexylnitrone (32). Yellow solid (86%); mp 194.0−196.0 °C. ^{1}H NMR (CDCl$_3$) δ: 1.21−1.50 (m, 5H), 1.99−2.20 (m, 5H), 4.16 (m, 1H), 7.52−7.47 (m, 3H), 8.34−8.31 (m, 2H), 8.51 (s, 1H). ^{13}C NMR (CDCl$_3$) δ: 175.4 (C5), 172.2 (C3), 133.4 (Cq), 130.6 (Co), 129.1 (Cm), 128.5 (Cp), 128.0 (CH=N), 74.9 (*C*CH$_2$), 31.7 (*C*CH$_2$), 25.3 (CH$_2$*C*H$_2$). EI-MS, *m/z* (abundance, %): 287 (M$^{+\bullet}$, 79), 269 (32), 243 (25), 205 (86), 189 (31), 135 (60), 103 (42), 55 (100). IR (KBr) ν: 1551, 1465, 1432, 1352, 926. Anal. Calcd for (C$_{15}$H$_{17}$N$_3$OS): C, 62.69; H, 5.96; N, 14.62; S, 11.16. Found: C, 62.39; H, 5.78; N, 14.55; S, 10.98.

α(Z)-(3-Phenyl-1,2,4-thiadiazol-5-yl)-*N*-benzylnitrone (33). White solid (65%); mp 156.0−158.0 °C. ^{1}H NMR (CDCl$_3$) δ: 5.21 (s, 2H), 7.46−7.45 (m, 3H), 7.48 (s, 5H), 8.30−8.27 (m, 2H), 8.35 (s, 1H). ^{13}C NMR (CDCl$_3$) δ: 174.9 (C5), 171.9 (C3), 132.8 (Cq), 130.9 (Cq), 130.3 (Co), 129.9 (Cm), 128.8 (Co), 129.5 (Cm), 129.4 (Cp), 128.7 (Cp), 128.0 (CH=N), 69.5 (CH$_2$). EI-MS, *m/z* (abundance, %): 295 (M$^{+\bullet}$, 32), 278 (4), 135 (5), 103 (7), 91 (100). IR (KBr) ν: 1534, 1465, 1423, 1323, 1234, 1130, 919, 726. Anal. Calcd for (C$_{16}$H$_{13}$N$_3$OS): C, 65.06; H, 4.44; N, 14.23; S, 10.86. Found: C, 64.88; H, 4.30; N, 14.12; S, 10.61.

α(Z)-[3-(4-Chlorophenyl)-1,2,4-thiadiazol-5-yl]-*N-tert*-butylni- trone (34). Yellow solid (68%); mp 129.0−131.0 °C. ^{1}H NMR (CDCl$_3$) δ: 1.68 (s, 9H), 7.46 (d, 2H, *J* = 8.5 Hz), 8.27 (d, 2H, *J* = 8.5 Hz), 8.63 (s, 1H). ^{13}C NMR (CDCl$_3$) δ: 175.7 (C5), 170.7 (C3), 136.2 (Cp), 131.5 (Cq), 129.3 (Co), 128.9 (Cm), 125.8 (CH=N), 71.7 (*C*(CH$_3$)$_3$), 28.1 (C(*C*H$_3$)$_3$). EI-MS, *m/z* (abundance, %): 295 (M$^{+\bullet}$, 26), 239 (35), 169 (8), 137 (14), 57 (100). IR (KBr) ν: 1548, 1471, 1420, 1404, 1346, 1236, 1118, 913, 838, 741, 558. Anal. Calcd for (C$_{13}$H$_{14}$ClN$_3$OS): C, 52.79; H, 4.77; N, 14.21; S, 10.84. Found: C, 52.78; H, 5.07; N, 14.02; S, 11.01.

α(Z)-[3-(4-Methoxyphenyl)-1,2,4-thiadiazol-5-yl]-*N-tert*-butylni- trone (35). Orange solid (88%); mp 130.0−132.0 °C. ^{1}H NMR (CDCl$_3$) δ: 1.67 (s, 9H), 3.87 (s, 3H), 7.01 (d, 2H, *J* = 8.7 Hz), 8.28 (m, 2H, *J* = 8.8 Hz), 8.63 (s, 1H). ^{13}C NMR (CDCl$_3$) δ: 175.4 (C5), 171.6 (C3), 161.2 (Cp), 129.5 (Co), 125.9 (CH=N), 114.0 (Cm), 71.6 (*C*(CH$_3$)$_3$), 55.3 (OCH$_3$), 28.0 (C(*C*H$_3$)$_3$). EI-MS, *m/z* (abundance, %): 291 (M$^{+\bullet}$, 91), 235 (100), 165 (26), 133 (38), 57 (89). IR (KBr) ν: 1609, 1547, 1408,1314, 1246, 1180, 1026, 838, 750, 659, 556. Anal. Calcd for (C$_{14}$H$_{17}$N$_3$O$_2$S): C, 57.71; H, 5.88; N, 14.42; S, 11.00. Found: C, 56.56; H, 5.68; N, 14.23; S, 10.80.

α(Z)-[3-(3,5-Di-*tert*-butyl-4-hydroxyphenyl)-1,2,4-thiadiazol-5- yl]-*N-tert*-butylnitrone (36). Yellow solid (92%); mp 292.0−294.0 °C. ^{1}H NMR (CDCl$_3$) δ: 1.51 (s, 18H), 1.68 (s, 9H), 5.48 (s, 1H), 8.19 (s, 2H), 8.64 (s, 1H). ^{13}C NMR (CDCl$_3$) δ: 175.2 (C5), 172.7 (C3), 155.9 (Cp), 136.1 (Cq), 126.0 (Co), 125.2 (CH=N), 124.6 (Cm), 71.5 (*C*(CH$_3$)$_3$), 34.4 (*C*(CH$_3$)$_3$), 30.3 (C(*C*H$_3$)$_3$), 28.4 (C(*C*H$_3$)$_3$). EI-MS, *m/z* (abundance, %): 389 (M$^{+\bullet}$, 87), 374 (46), 333 (36), 318 (100), 57 (40). IR (KBr) ν: 3593, 2961, 1544, 1398, 1254, 1236, 1121, 913, 747, 696, 555. Anal. Calcd for (C$_{21}$H$_{31}$N$_3$O$_2$S): C, 64.75; H, 8.02; N, 10.79; S, 8.23. Found: C, 64.50; H, 8.30; N, 10.63; S, 8.18.

α(Z)-(4-Methyl-1,2,3-thiadiazol-5-yl)-*N-tert*-butylnitrone (37). Beige solid (77%); mp 154.0−156.0 °C. ^{1}H NMR (CDCl$_3$) δ: 1.67 (s, 9H), 2.85 (s, 3H), 8.20 (s, 1H). ^{13}C NMR (CDCl$_3$) δ: 157.7 (C5), 138.4 (C4), 122.7 (CH=N), 71.2 (*C*(CH$_3$)$_3$), 28.5 (C(*C*H$_3$)$_3$), 13.9 (CH$_3$). EI-MS, *m/z* (abundance, %): 199 (M$^{+\bullet}$, 2), 171 (2), 123 (30), 108 (42), 57 (100). IR (KBr) ν: 3136, 2997, 2972, 1547, 1493, 1439, 1361, 1323, 1201,1140, 1035, 887, 827, 723, 572. Anal. Calcd for (C$_8$H$_{13}$N$_3$O$_2$S): C, 48.22; H, 6.58; N, 21.09; S, 16.09. Found: C, 48.09; H, 6.30; N, 20.82; S, 15.88.

α(Z)-(5-Ethoxycarbonyl-1,2,3-thiadiazol-4-yl)-*N-tert*-butylnitro- ne (38). Yellow solid (48%); mp 93.0−95.0 °C. ^{1}H NMR (CDCl$_3$) δ: 1.44 (t, 3H), 1.65 (s, 9H), 4.48 (q, 2H), 8.52 (s, 1H). ^{13}C NMR (CDCl$_3$) δ: 159.9 (C=O), 153.9 (C4), 143.2 (C5), 121.2 (CH=N), 72.8 (*C*(CH$_3$)$_3$), 63.3 (*C*H$_2$CH$_3$), 28.5 (C(*C*H$_3$)$_3$), 14.5 (CH$_2$*C*H$_3$). EI-MS, *m/z* (abundance, %): 257 (M$^{+\bullet}$, 1), 201 (9), 173 (9), 127 (6), 57 (100). IR (KBr) ν: 3147, 299, 1732, 1562, 1535, 1394, 1313, 1209, 1153, 1087, 910, 860, 767, 559. Anal. Calcd for (C$_{10}$H$_{15}$N$_3$O$_3$S): C, 46.68; H, 5.88; N, 16.33; S, 12.46. Found: C, 46.33; H, 5.50; N, 16.20; S, 12.19.

α(Z)-(4-Phenylfuroxan-3-yl)-*N-tert*-butylnitrone (39). Yellow solid (57%); mp 78.0−80.0 °C. ^{1}H NMR (CDCl$_3$) δ: 1.61 (s, 9H), 7.42−7.56 (m, 5H), 7.58 (s, 1H). ^{13}C NMR (CDCl$_3$) δ: 155.8 (C4), 133.9 (Cm), 130.5 (Co), 126.7 (Cp), 114.9 (CH=N), 110.3 (C3), 73.2 (*C*(CH$_3$)$_3$), 28.3 (C(*C*H$_3$)$_3$). EI-MS, *m/z* (abundance, %): 261 (M$^{+\bullet}$, 2), 205 (18), 145 (10), 105 (18), 57 (100). IR (KBr) ν: 3138, 2979, 2935, 1600, 1560, 1456, 1382, 1247, 1199, 1139, 1028, 983, 858, 812, 709, 556. Anal. Calcd for (C$_{13}$H$_{15}$N$_3$O$_3$): C, 59.76; H, 5.79; N, 16.08. Found: C, 59.45; H, 5.61; N, 15.87.

α(Z)-(3-Methylfuroxan-4-yl)-*N-tert*-butylnitrone (40). Yellow solid (81%); mp 119.0−121.0 °C. ^{1}H NMR (CDCl$_3$) δ: 1.61 (s,

9H), 2.33 (s, 3H), 7.96(s, 1H). ^{13}C NMR (CDCl$_3$) δ: 151.1 (C4), 117.9 (CH=N), 112.6 (C3), 72.9 ($\underline{C}$(CH$_3$)$_3$), 28.6 (C($\underline{C}$H$_3$)$_3$), 10.4 (CH$_3$). EI-MS, *m/z* (abundance, %): 199 (M$^{+\bullet}$, 3), 143 (9), 113 (6), 83 (4), 57 (100). IR (KBr) ν: 2984, 1616, 1566, 1457, 1364, 1251, 1178, 1140, 834, 763, 549. Anal. Calcd for (C$_8$H$_{13}$N$_3$O$_3$): C, 48.23; H, 6.58; N, 21.09. Found: C, 48.02; H, 6.30; N, 20.92.

α(Z)-(Benzofuroxan-5(6)-yl)-*N-tert*-butylnitrone (41). Yellow solid (61%); mp 112.0−114.0 °C. ^{1}H NMR (CDCl$_3$) δ: 1.60 (s, 9H), 7.32 (bs, 1H), 7.50 (bs, 1H), 7.60 (s, 1H), 9.33 (bs, 1H). ^{13}C NMR (CDCl$_3$) δ: 127.4 (CH=N), 72.5 ($\underline{C}$(CH$_3$)$_3$), 28.3 (C($\underline{C}$H$_3$)$_3$). EI-MS, *m/z* (abundance, %): 235 (M$^{+\bullet}$, 9), 179 (17), 163 (5), 152 (6), 57 (100). IR (KBr) ν: 2984, 1610, 1570, 1487, 1361, 1236, 1153, 1014, 868, 828, 585. Anal. Calcd for (C$_{11}$H$_{13}$N$_3$O$_3$): C, 56.16; H, 5.57; N, 17.86. Found: C, 55.99; H, 5.34; N, 17.70.

Measurement of Cell Viability. Culture of SH-SY5Y Cells. SH-SY5Y cells, at passages between 3 and 16 after defreezing, were maintained in a minimum essential medium (MEM) containing 15 nonessential amino acids (NEAAs) and supplemented with 10% fetal bovine serum, 1 mM glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin (reagents from GIBCO, Madrid, Spain). Cultures were seeded into flasks containing supplemented medium and maintained at 37 °C in 5% CO$_2$/humidified air.

Cell Viability Assays. The effects of nitrones on SH-SY5Y cell viability were determined by measuring the activity of LDH released into the culture medium (Cytotox 96 LDH assay, Promega, U.K.). The assay was performed in accordance with the manufacturer's protocol using SH-SY5Y cells seeded at 10^4 cells/well in 96-well plates. Cells were exposed to 10, 1, and 0.05 μM of each one nitrone for 24 h.

The effects of H$_2$O$_2$ on SH-SY5Y cell viability were determined by measuring the activity of LDH released into the culture medium (Cytotox 96 LDH assay, Promega, U.K.). The assay was performed in accordance with the manufacturer's protocol using SH-SY5Y cells seeded at 10^4 cells/well in 96-well plates. Cells were exposed to 10 μM of each one heteroarylnitrone for 1 h and subsequently exposed to 100 μM H$_2$O$_2$ for 24 h. An amount of 5 mM NAC was used as positive test.

Unspecific Mammalian Cytotoxicity. J774 murine macrophage-like cells (ATCC) were maintained by passage in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine and supplemented with 10% heat-inactivated fetal calf serum. J774 cells were seeded (1 × 10^5 cells/well) in 96-well microplates with 100 μL of RPMI medium supplemented with fetal serum. Cells were allowed to attach for 48 h in a humidified 5% CO$_2$/95% air atmosphere at 37 °C and then exposed to nitrones (50−500 μM) for 48 h. Afterward, cell viability was assessed by measuring the mitochondrial-dependent reduction of 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) to formazan. For that purpose, MTT was added to cells to a final concentration of 0.2 mg/mL and cells were incubated at 37 °C for 3 h. After removal of the media, formazan crystals were dissolved in DMSO, and the absorbance at 560 nm was read using a microplate spectrophotometer. Results are expressed as IC$_{50}$ (compound concentration that reduce 50% control absorbance at 560nm). Every IC$_{50}$ is the average of two different experiments.

Blood−Brain Barrier Permeation Assay. Brain penetration of new nitrones was evaluated using a parallel artificial membrane permeation assay (PAMPA) in a similar manner as described previously.[27] Commercial drugs were purchased from Sigma, Aldrich, Acros, and Fluka. Millex filter units (PVDF membrane, diameter 25 mm, pore size 0.45 μm) were acquired from Millipore. The porcine brain lipid (PBL) was obtained from Avanti Polar Lipids. The donor microplate was a 96-well filter plate (PVDF membrane, pore size 0.45 μm), and the acceptor microplate was an indented 96-well plate, both from Millipore. The acceptor 96-well microplate was filled with 170 μL of PBS/ethanol (9:1), and the filter surface of the donor microplate was impregnated with 4 μL of PBL in dodecane (20 mg/mL). Compounds were dissolved in PBS/ethanol (9:1) at 1 mg/mL, filtered through a Millex filter, and then added to the donor wells (170 μL). The donor filter plate was carefully put on the acceptor plate to form a sandwich, which

was left undisturbed for 120 min at 25 °C. After incubation, the donor plate was carefully removed and the concentration of compounds in the acceptor wells was determined by UV spectroscopy. Every sample was analyzed in four wells and at least in three independent runs, and the results are given as the mean ± standard deviation.

Determination of Antioxidant Activity. OFRAC$_{FL}$ Assay. A luminescence spectrometer LS 50B (PerkinElmer, Boston, MA), a heating circulator bath DC1-B3 (Haake Haake Fisons, Karlsruhe, Germany), and quartz cuvettes were used. For the ORAC$_{FL}$ assay, the 490-P excitation and 515-P emission filters were used, and the fluorescence measurement was carried out at 60 °C. The method of Ou et al.[29] was modified as follows: the reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction volume was 3000 μL. Studied compounds (10−1000 μM final concentrations) and fluorescein (215 μL, 70 nM final concentration) solutions were placed in the quartz cuvette. The mixture was preincubated for 30 s at 60 °C. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) solution (240 μL; 12 mM, final concentration) was added rapidly using a single channel pipet. The quartz cuvette was immediately placed in the luminescence spectrometer and the fluorescence recorded every minute for 12 min. As a blank FL + AAPH in phosphate buffer instead of the studied compounds solutions were employed, and eight calibration solutions using Trolox (1−8 μM, final concentration) as antioxidant positive control were also carried out in each assay. All the reaction mixtures were prepared in duplicate, and at least three independent assays were performed for each sample. Raw data were exported to an OriginPro (OriginLab Corporation, Northampton, MA) sheet for further calculations. Blank and antioxidant curves (fluorescence versus time) were first normalized by dividing original data by fluorescence at $t = 0$ s. From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as

$$AUC = 1 + \sum_{i=1}^{i=12} \frac{f_i}{f_0}$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i. The net AUC corresponding to each sample was calculated by subtracting the AUC corresponding to the blank. Regression equations between net AUC and antioxidant concentration were calculated for all the samples. ORAC$_{FL}$ values were expressed as PBN equivalents by using the standard curve calculated for each assay. Final results were expressed in μmole of PBN equivalent per μmole of samples.

DPPH Assay. The reaction was carried out in 96-well microplates, and the final reaction mixture was 100 μL per well. Antioxidant (0.1 mM, final concentration) and DPPH (0.1 mM, final concentration) were dissolved in EtOH and preincubated for 5 min at 37 °C, and the absorbance at 520 nm was read using a microplate spectrophotometer. The antioxidant activity was determined as the RP of DPPH, calculated as follows: RP = 100 [($A_0 - A_c$)/A_0], where A_0 is the untreated DPPH absorbance and A_c is the value for added sample concentration c.

ESR Spectroscopy. The spin trapping ability of the developed heteroarylnitrones was studied by ESR spectroscopy. ESR spectra were recorded in the X band (9.85 GHz) using a Bruker ECS 106 spectrometer with a rectangular cavity and 50 kHz field modulation. All the spectra were registered in the same scale after 15 scans. The hyperfine splitting constants were estimated to be accurate within 0.05 G.

Generation of Free Radicals. Hydroxyl radical ($^\bullet$OH) was generated from a standard Fenton system: Fe(NH$_4$)$_2$(SO$_4$)$_2$·6H$_2$O (50 μL, 0.5 mM) was added to a solution of nitrone (50 mM, final concentration) and H$_2$O$_2$ (50 μL, 0.5 mM) in phosphate buffer (final reaction mixture was 250 μL). Cysteinyl radical (SO$_3$$^{\bullet-}$) was generated from a standard Fenton system: Fe(NH$_4$)$_2$(SO$_4$)$_2$·6H$_2$O (50 μL, 0.5 mM) was added to a solution of nitrone (50 mM, final concentration), Na$_2$SO$_3$ (50 μL, 250 mM), and H$_2$O$_2$ (50 μL, 0.5 mM) in phosphate buffer (final reaction mixture was 250 μL).

Ethanol radical (CH$_3$$^\bullet$CHOH) was generated from a standard Fenton system: Fe(NH$_4$)$_2$(SO$_4$)$_2$·6H$_2$O (50 μL, 0.5 mM) was added to a solution of nitrone (50 mM, final concentration), EtOH (50 μL), and H$_2$O$_2$ (50 μL, 0.5 mM) in phosphate buffer (final reaction mixture was 250 μL).

Supporting Information Available: Elemental analysis results for heteroarylnitrone derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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