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New heteroaryl nitrones with spin trap properties: Identification of a 4-furoxanyl derivative with excellent properties to be used in biological systems

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ABSTRACT

A new series of heteroaryl nitrones, **1**–**7**, bearing furoxanyl and thiadiazolyl moieties, were evaluated for their free radical-trapping properties. The physicochemical characterization by electron paramagnetic resonance (EPR) demonstrated its capability to trap and stabilize oxygen-, carbon-, sulfur-, and nitrogen-centered free radicals. The 4-furoxanyl nitrone **3** (**FxBN**), α(*Z*)-(3-methylfuroxan-4-yl)-*N*-*tert*-butylnitrone, showed appropriate solubility in aqueous solution and taking into account that this physicochemical property is very important for biological applications, we studied it deeply in terms of its trapping and kinetic behaviors. For this, kinetic studies of the hydroxyl adduct decay gave rate constants k_{ST} of 1.22×10^{10} dm^3 mol^{-1} s^{-1} and half-live up to 7200 s at physiological pH, without any artifactual signals. The ability of **FxBN** to directly traps and stabilizes superoxide free radical, with a half-life of 1620 s at physiological pH, was also demonstrated. Besides, **FxBN**-hydroxyl and -superoxide adducts exhibited distinct and characteristic EPR spectral patterns. Finally, we confirmed the ability of **FxBN** to act as spin trap in a specific biological system, that is, in the free radical production of experimental anti-trypanosomatid drugs using *Trypanosoma cruzi* microsomes as biological system. Moreover, previous observations of low **FxBN** toxicity transform it in a good candidate for in vivo spin trapping.

1. Introduction

Reactive oxygen (ROS) and nitrogen species (RNS) are produced as natural metabolic byproducts in the human body.[1,2] These species could be mediators in various physiological oxidative processes and neurodegenerative diseases such as Alzheimeŕs and Parkinsoń diseases.[3,4] Besides, several drugs (currently used or proposed to use) act through free radical generation during their metabolism or mechanism of action.[5,6] In the last years spin trapping technique has been applied to characterize and understand the role played by both oxygen and nitrogen-centered free radicals involved in biological processes.[7,8]

In this sense, the spin trapping technique has proved to be a useful and powerful tool in the study of highly reactive free radical species.[9–11] The most widely used spin trapping agents have been for a long time the non-cyclic nitrones α-phenyl-*N*-*t*-butylnitrone and α-4-pyridyl-1-oxide-*N*-*tert*-butyl nitrone (PBN and POBN, respectively, Fig. 1) and the cyclic one 5,5-dimethylpyrroline-*N*-oxide (DMPO, Fig. 1).[12–15] These available spin traps present many disadvantages such as low water solubility, sensitivity to nucleophilic attack of water or others degradation reactions and low stability of the spin adduct formed.[10,16] Several new spin traps with different trapping abilities and properties like DEPMPO, BMPO, and EMPO[17,18] have been developed, which would be used

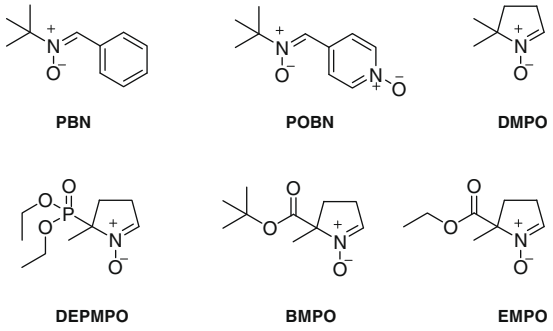

Figure 1. Structures of various nitrones commonly used as spin trap agents.

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specially for trapping superoxide radical (Fig. 1). However, these new spin traps are not completely efficient for all free radical species. Recently, the syntheses of a new family of spin trap of EMPO derivatives have shown that their spin adducts present reasonable stability.[19,20] By the other side, a recent review of the most used spin traps for superoxide radical has concluded that the proper choice of the trap depends on not only the experimental conditions but also the kind of data to obtain from the analysis.[21]

On the other hand, the development of new spin traps derived from PBN has showed a wide variety of applications including neuroprotective and antioxidant activities so as free radical scavenger properties.[22–26] Recently, we described the interesting antioxidant and neuroprotective activities of a series of novel nitrone containing heteroaryl derivatives structurally relates to PBN, nitrones **1**–**7** (Fig. 2).[27] Moreover, these nitrones were not cytotoxic to SH-SY5Y neuronal cells and J-774 macrophages[27] transforming them in excellent chemical hits to be studied as spin trap entities for biological systems.

In this work we study the spin trapping capabilities of some selected heteroaryl nitrones from the first developed series[27] against oxygen-, carbon-, sulfur-, and nitrogen-centered free radicals, for example, hydroxyl, superoxide, 1-hydroxyethyl, methyl, sulfur trioxide anion, and azidyl free radicals. The optimum aqueous solubility of **FxBN** together with its ease synthetic procedure,[27] conducts us to analyze its capability to act as spin trap for oxygen-centered free radicals in biological system. Consequently, kinetic constants and decay rates were obtained for **FxBN**-hydroxyl and -superoxide adducts at pH 7.4 in phosphate buffer. Additionally, we used **FxBN** as spin trap for well known anti-trypanosomatid drugs that produce hydroxyl and drugs' free radicals in presence of *Trypanosoma cruzi* (*T. cruzi*) microsomes as biological model.

2. Methods and results

2.1. Chemistry

Figure 2 illustrates the new developed heteroaryl nitrones studied herein as spin traps. They were synthesized through the condensation between heteroaromatic aldehydes and *N*-*tert*-butyl hydroxylamine with good to excellent yields, as previously described.[27] The studied heteroaryl nitrones were (Fig. 2) α(*Z*)-(5-phenyl-1,2,4-thiadiazol-3-yl)-*N*-*tert*-butylnitrone (**1**), α(*Z*)-(3-phenyl-1,2,4-thiadiazol-5-yl) -*N*-*tert*-butylnitrone (**2**), α(*Z*)-(3-methylfuroxan-4-yl)-*N*-*tert*-butylnitrone (**3**, **FxBN**), α(*Z*)-(4-phenylfuroxan-3-yl)-*N*-*tert*-butylnitrone (**4**), α(*Z*)-(benzofuroxan-5(6)-yl)-*N*-*tert*-butylnitrone (**5**), α(*Z*)-(5-ethoxycarbonyl-1,2,3-thiadiazol-4-yl)-*N*-*tert*-butylnitrone (**6**), α(*Z*)-(4-methyl-1,2,3-thiadiazol-5-yl)-*N*-*tert*-butylnitrone (**7**).

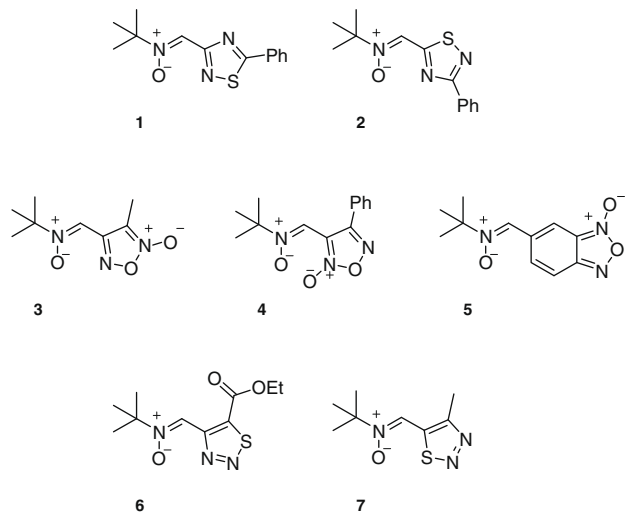


Figure 2. Structures of the new heteroaryl nitrones **1**–**7** studied as new spin traps.

2.2. Spin trapping experiments

Firstly, the spin trapping properties of the new nitrones were investigated against oxygen-, carbon- and sulfur-centered free radicals produced by hydroxyl, 1-hydroxylethyl and sulfur trioxide anion radicals. These radicals were generated by Fenton's reagents, using an aqueous Fe^{2+} solution and an aqueous solution of hydrogen peroxide (1%), ethanol or sodium sulfite, respectively. The EPR spectra were simulated allowing us a systematic study on the dependencies of the spectral features with some magnetic parameters and accurate parameter extraction from experimental spectra. Except 3-furoxanyl nitrone **4** each of the heteroaryl nitrones tested gave an EPR-detectable spin adduct with the hydroxyl radical (Fig. 3). In the same conditions, DMPO gave the typical strong quartet signal as results of the DMPO-·OH adduct formation (data not shown). The EPR spectrum of the resulting spin adducts were composed of six-line (triplet of doublet) due to coupling of the unpaired electron with nitrogen and hydrogen at alpha carbon. The 3-furoxanyl nitrone **4** was the only one that did not give a detectable spin adduct with the very reactive ·OH free radical. This could be explained as the result of the well known ring opening susceptibility of this kind of furoxan derivatives[28,29]-substituted in position 3 by an electron-withdrawing group, here the nitrone moiety. In this case ·OH free radical could initiate, through the cyclic nitrone (furoxan carbon 3), a ring opening process that yield non-electron unpaired entities. The 1-hydroxylethyl spin adduct was observed for the nitrones **2**–**7** gave a six-line spectrum (Fig. 4) with strong EPR signal. The performed EPR simulations correlated well with experimental EPR spectra. Finally, the spin trapping properties of the new heteroaryl nitrones were investigated against sulfur-centered free radical produced by sulfur trioxide anion free radical (Fig. 5). Furoxanyl nitrones **FxBN**[27] and **4**, and 1,2,3-thiadiazolyl nitrones **6**[27] and **7** gave a strong EPR signal explained in terms of triplet of doublet. The 1,2,4-thiadiazolyl nitrone **2**[27] gave a weak signal while no signal was observed for 1,2,4-thiadiazolyl nitrone **1** and benzofuroxanyl nitrone **5**. In all the cases, the spin adducts from heteroaryl nitrones showed symmetric patterns. Table 1 resume the hyperfine constant coupling for the different generated spin adducts. In the case of nitrones **FxBN** and **6**, the nitrogen hyperfine constants between the trapping of the hydroxyl, ethanol and sulfite radical have the same values, although these showed slight difference in the hyperfine constants of the hydrogen atom. It can be clearly appreciated that **FxBN** and 1,2,3-thiadiazolyl nitrone **7** have very different hyperfine coupling constants between the trapping of the hydroxyl and ethanol radical but both nitrones showed similar hyperfine constants for the sulfite-adduct radicals. 4-Furoxanyl nitrone **FxBN** and 1,2,3-thiadiazolyl nitrones **6** and **7** were able to produce clear signals with the three different studied free radical, that is, oxygen-, carbon-, and sulfur-centered free radical.

Due to 4-furoxanyl nitrone **FxBN** has better aqueous solubility than the other heteroaryl nitrones developed we studied it against other spin species, oxygen-, carbon- and nitrogen-centered free radicals. Superoxide was generated using the xanthine/xanthine oxidase couple whereas methyl and azidyl radicals were produced by Fenton reaction with dimethylsulfoxide and sodium azide, respectively. Table 2 gathers the corresponding hyperfine constants and Figure 6 shows the well define EPR spectra. **FxBN** gave an EPR-detectable spin adduct with the superoxide radical exhibiting an unsymmetrical spectra with nine-lines, with higher intensity than the EPR spectra of DMPO–superoxide adduct. The EPR spectrum with methyl radical was composed of triplet of doublet due to cou-

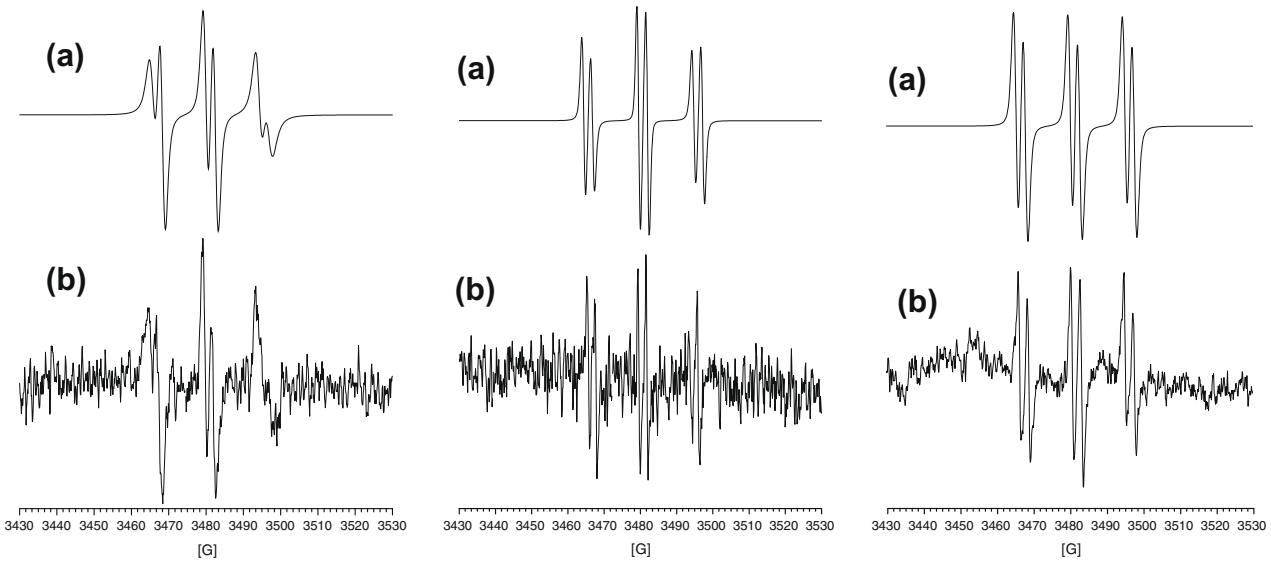


Figure 3. EPR spectra of heteroaryl nitrones **1**-, **5**- and **7**-hydroxyl free radical adducts. (a) Simulated spectra; (b) experimental spectra. For EPR spectra with heteroaryl nitrones **2**, **FxBN** and **6** see Ref. 27.

pling with nitrogen atom (triplet) and β-hydrogen atom (doublet) at α-carbon atom. The spin adduct with the azidyl radical exhibit twelve-line attributed to a triplet of triple doublet due to two different nitrogen atoms, one from the nitrone and the other from the azidyl radical, and to the β-hydrogen atom belonging to the nitrone trap with the last hyperfine constant coupling similar (Table 2).

2.3. Spin adduct stability and determination of the decay rate constant for FxBN-adducts

In spite of this, all nitrones under study showed a good behavior as spin traps, 4-furoxanyl nitrone **FxBN** has excellent solubility in aqueous milieu which is an advantage to apply in biological experiments. To get insight into the physicochemistry properties of **FxBN**-·OH adduct stability and kinetic studies in phosphate buffer (pH 7.4) were performed. The spin adducts stability was studied following the disappearance of the EPR signal intensity of **FxBN**-·OH adduct (Fig. 7a). The mechanism of the decay could be attributed to a solvolysis process.[14,30,31] The half-life of **FxBN**-·OH adduct was determined assuming that the decay is of first order. This furoxanyl nitrone **FxBN** have excellent stability with a $t_{1/2}$ more than twofold higher the value for DMPO-·OH adduct, $t_{1/2}$ = 55 min in neutral milieu,[7] and 200-fold higher than the value for PBN-·OH adduct, $t_{1/2}$ = 0.63 min (Table 3).[14,32] On the other hand, it is very well known the use of DMPO in detecting superoxide free radical, which is based on the relative stability of the adduct and its characteristic EPR spectrum, however the half-life in neutral milieu is only about 1 min, therefore the intensity of the EPR signal is not proportional to the amount of superoxide generated in the system.[7,16] For this reason we decided to determinate the half-life of the **FxBN**–superoxide adduct in neutral milieu. Figure 7b shows the decay time course recorded for **FxBN**–superoxide adduct, at pH 7.4. The half-life of **FxBN**–superoxide adduct in neutral milieu, extracted from the slope, is near to 30-fold higher the corresponding value for DMPO-O^{2-} adduct (Table 3).

2.4. Competitive ·OH-trapping studies

In a competitive study we determined the ratio of constants (k_2/k_1) of hydroxyl radical for **FxBN** or PBN and for DMPO (Table 3), being for DMPO $k_1 = 3.6 \times 109\ dm^3\ mol^{-1}\ s^{-1}$.[36] It is possible to observe that **FxBN** exhibits a fast rate in trapping hydroxyl radical as compared with DMPO (Fig. 8a). However, when the same kind of competitive experiment was performed with 1,2,3-thiadiazolyl nitrone **7** a slower trapping process than DMPO occurred (Fig. 8b). This different reactivity and stability could be attributed to the different structural heterocyclic motives between both nitrones[37] claiming the relevance of the 4-furoxanyl-substructure on **FxBN** in the spin stabilization.

2.5. Spin trapping in biological systems studies

In the last years, it has been proposed in several studies that antichagasic drugs, as nitroaromatic compounds or *N*-oxide heterocyclic derivatives, could act by generation of free radical toxic species through a redox-cycling process by bioreduction of the nitro or *N*-oxide group during its metabolization.[5,38–40] Due to the great potential found in the spin trapping properties of **FxBN**, in vitro biological spin trapping experiments were performed using *T. cruzi*, the etiologic agent of Chagas disease, as model. In this sense, two different nitro compounds, NF and $PtCl_2$(NF),[41,42] and two different benzofuroxan (benzo[1,2-*c*]1,2,5-oxadiazole *N*-oxide) derivatives, TBfx-1 and TBfx-2,[6] which are well known hydroxyl and drugs' free radicals producers in presence of *T. cruzi* parasite, were studied. In the experimental assayed conditions the 4-furoxanyl nitrone **FxBN** was capable to act exclusively as hydroxyl spin trap in this biological system as it is shown in Figure 9. The EPR spectra for each of the four tested compounds exhibited six-line (triplet of doublet), with moderate to high intensity, in agreement with the **FxBN**–hydroxyl adduct.[27]

3. Discussion and conclusions

The novel developed heteroaryl nitrones are analogues of PBN (Fig. 1) where the phenyl group was substituted by different heterocyclic systems. In 2006, Nepveu and co-workers described a series of imidazolyl nitrones, where PBN-phenyl group was substituted by different imidazole moieties, with excellent spin trap properties as result of the presence of this heterocycle.[43] In our case, we investigated four different hetero-aromatic systems instead of PBN-phenyl group, that is, 1,2,4-thiadiazole in nitrone

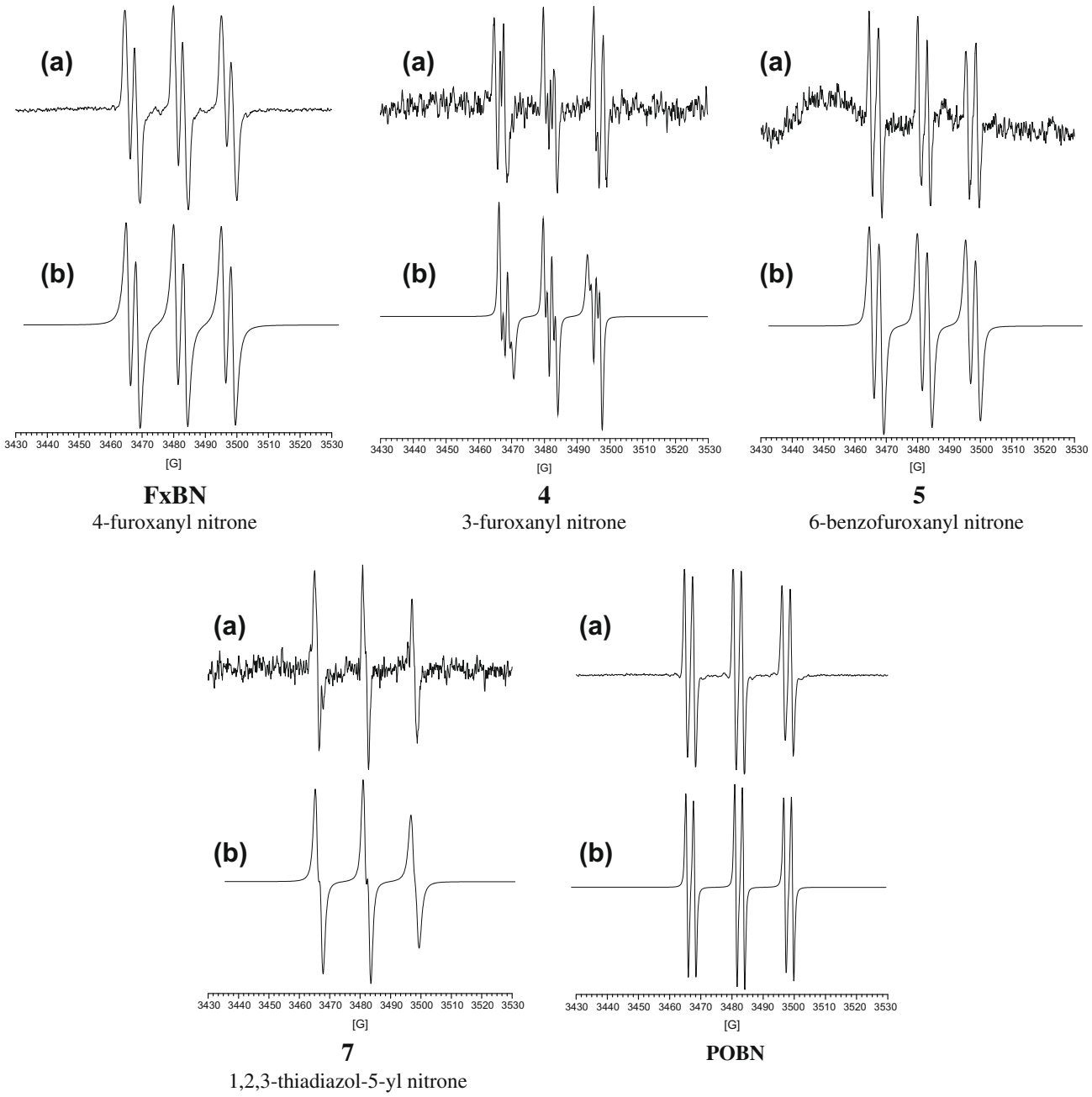
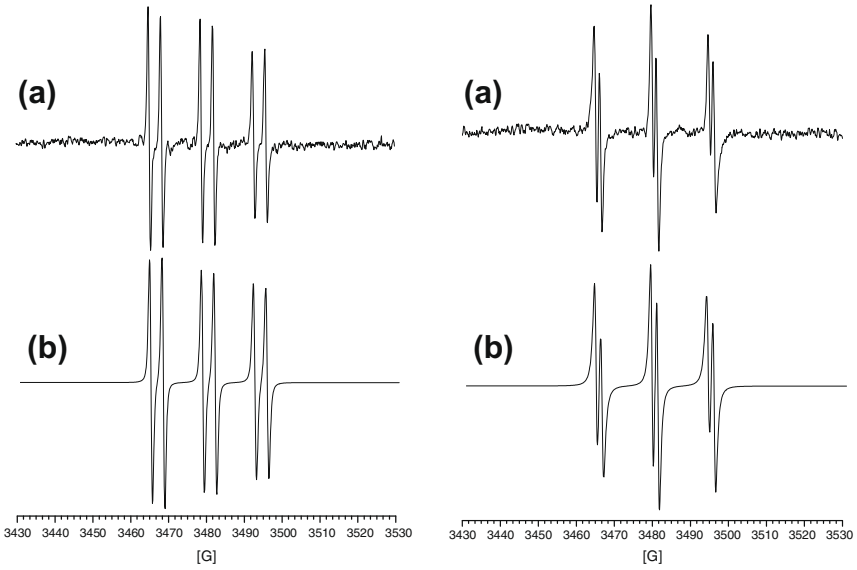


Figure 4. EPR spectra of heteroaryl nitrones **FxBN**-, **4**-, **5**- and **7**-1-hydroxylethyl free radical adducts. Spectrum of POBN-1-hydroxylethyl free radical adduct is included as reference. (a) Experimental spectra; (b) simulated spectra. For EPR spectra with heteroaryl nitrones **2**, and **6** see Ref. 27.

1 and **2**, 1,2,5-oxadiazole *N*-oxide in **FxBN** and **4**, benzo[1,2-*c*]1,2,5-oxadiazole *N*-oxide in nitrone **5**, and 1,2,3-thiadiazole in nitrone **6** and **7** (Fig. 2). These derivatives were selected from our nitrone-chemical library attempting to study the influence of the heteroatoms and the heterocyclic substituents positions in the solubility and in the spin trapping properties. In this sense, we selected heterocycles porting S and N (nitrones **1**–**2** and **6**–**7**), and N and O (nitrones **3**–**5**) in different relative positions. On the other hand, we studied different heterocyclic-relative positions of the nitrones moieties, for example, positional isomers **1** and **2**, or 4- and 3-furoxanylnitrones **FxBN** and **4**, respectively.

These new heteroaryl nitrones have demonstrated that present a high capacity to trap and stabilize oxygen-, carbon-, sulfur-, and nitrogen-centered radicals. Superoxide $O_2^{\cdot-}$ is often considered to be the main radical of oxidative stress whereas the hydroxyl radical ·OH is considered to be the predominant radical contributing to injury to tissues or cells through peroxidation of their components and the carbon-centered species generally result from the oxidative attack of cell components.[44] In aqueous solution, we found that **FxBN** was able to form hydroxyl spin adduct with half-life higher than 2 h while the corresponding superoxide spin adduct has a half-life near to 0.5 h. Based in these values and the characteristic EPR spectrum obtained in this study, together with the ease synthetic procedure, the high aqueous solubility and the low toxicity of **FxBN**,[27] the conclusions that we could reach is that **FxBN** could be a better spin traps for oxygen radicals than PBN or DMPO in biological systems. In this sense, in the competition assays the results indicated that the new heteroaryl nitrone **FxBN** is more sensible that DMPO to trap hydroxyl free radical. Additionally, this 4-furoxanyl nitrone possesses spin trap capability in biological system and could be used without artifact as result of its low toxicity in the studied biological milieu.



4
3-furoxanyl nitrone

7
1,2,3-thiadiazol-5-yl nitrone

Figure 5. EPR spectra of heteroaryl nitrones **4**-, and **7**-sulfur trioxide anion radical adducts (50 mM). (a) Experimental spectra; (b) simulated spectra. For EPR spectra with heteroaryl nitrones **2**, **FxBN** and **6** see Ref. 27.

Table 1
Hyperfine constant coupling values, in G, of the different heteroaryl nitrones-radicals spin adducts

Compd	·OH		CH_3·CHOH		$·SO_3^{2-}$	
	a_N	a_H	a_N	a_H	a_N	a_H
1	14.8	2.4	(ns)[a]	(ns)	(ns)	(ns)
2	15.7[b]	0.9[b]	14.6	0.9	16.0	1.0
3 (FxBN)	15.2	1.9	15.1	2.5	15.2	1.5
4	(ns)	(ns)	14.5	4.0	13.9	4.0
5	14.0	2.2	15.6	3.0	(ns)	(ns)
6	15.2	3.3	15.2	3.0	15.3	4.0
7	14.5	2.6	14.6	0.9	15.0	1.8

[a] (ns): no signal.
[b] From Ref. 27.

Table 2
Detailed information for the trapping of oxygen-, carbon- and nitrogen-centered adduct radicals by **FxBN**

Hyperfine constant coupling (G)							
$·^-O_2$			$·CH_3$			$·N_3$	
a_N	a_H	a_H	a_N	a_H	a_N	a_N	a_H
15.6	1.9	1.2	16.0	3.1	14.4	2.2	2.6

Studies on the effect of functional group substitution at the C-3 position of 4-furoxanyl ring on spin trap reactivity toward

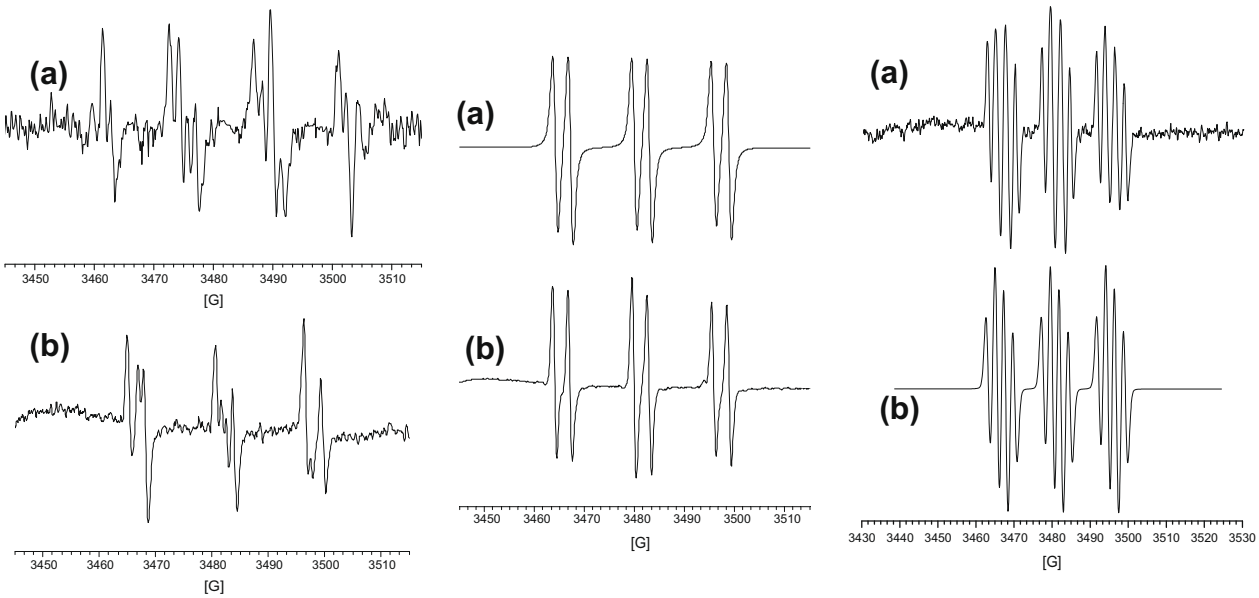


Figure 6. Left: (a) EPR spectra of DMPO–superoxide anion free radical adduct; (b) **FxBN**–superoxide anion free radical adduct. Centre: EPR spectrum of **FxBN**-methyl free radical adduct. (a) Simulated spectra; (b) experimental spectra. Right: EPR spectrum of **FxBN**-azidyl free radical adduct. (a) experimental spectra; (b) simulated spectra. All the spectra were acquired in the same experimental conditions and scale.

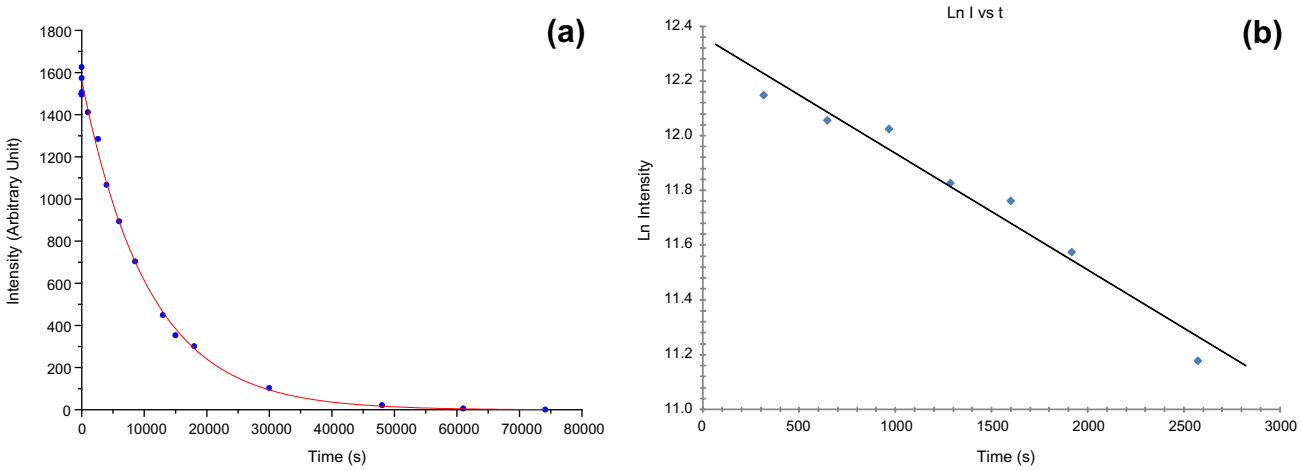


Figure 7. (a) Time course of the EPR signal intensity for **FxBN**-·OH adduct, in phosphate buffer (pH 7.4). The decay corresponds to a first order reaction. (b) Time course of the EPR signal intensity, expressed as Ln (intensity), for **FxBN**-O^{2-} adduct, in phosphate buffer (pH 7.4). The decay corresponds to a first order reaction.

Table 3
Half-life for **FxBN** and values of trapping rate constant and, DMPO and PBN with hydroxyl and superoxide free radicals

Nitrones	·OH adduct			$^{\cdot-}O_2$ adduct
	$t_{1/2}$ (s)	$k_{ST} \times 10^9$ (dm^3 mol^{-1} s^{-1})	k_2/k_1	$t_{1/2}$ (s)
FxBN	7560.0	12.2	3.39	1620.0
DMPO	3300.0	3.6	1.00	60.0[a]
PBN	36.0	2.6[b]	0.71	Not yet measured[c]

[a] For EMPO-O_2H adduct $t_{1/2}$ = 570.0 s, at pH 7.2.[33]
[b] From Ref. 34.
[c] From Ref. 35.

hydroxyl and superoxide free radicals, theoretical analysis and complementary EPR spectrum-simulation studies are in progress.

4. Experimental

4.1. Chemicals

The new developed heteroaryl nitrones were synthesized as previously described.[27] DMPO, POBN, dimethylsulfoxide, anhydrous sodium sulfite and monobasic potassium phosphate were purchased from Sigma–Aldrich. Sodium azide, hydrogen peroxide 30%, anhydrous ethanol and dibasic sodium phosphate heptahydrate were purchased from Merck. Ferrous ammonium sulfate hexahydrate was purchased from Mallinckrodt Baker.

4.2. Free radical generation and EPR measurements

The different studied radicals were generated by a Fenton type reaction using an aqueous Fe^{2+} solution, (aqueous ferrous ammonium sulfate hexahydrate, 1.0 mM), and an aqueous solution of hydrogen peroxide (1%) in phosphate buffer (pH 7.4) loaded in the EPR cell. In addition, for 1-hydroxyethyl-free radical was used anhydrous ethanol (250 mM), for methyl-free radical was used anhydrous dimethylsulfoxide (250 mM), for sulfur trioxide anion free radical was used anhydrous sodium sulfite (250 mM), and for azidyl free radical was used anhydrous sodium azide (250 mM). POBN (50 mM) was used as control to check the 1-hydroxylethyl free radical generation[45] and DMPO (50 mM) was used as control to check the hydroxyl and superoxide free radical generation. Superoxide anion free radical was generated using the xanthine/xanthine oxidase couple and the detection procedure was modified from reference[46] as follow: DMPO (50 mM) or **FxBN** (50 mM) was incubated with xanthine (0.5 mM) and xanthine oxidase (0.2 units/mL) in phosphate buffer (100 mM, pH 7.4, containing 1 mM DTPA). In the other experiments, solutions of nitrones **1**, **2** and **4**–**7** (50 mM, final concentration) in phosphate buffer/acetonitrile (70:30, v/v) or solution of **FxBN** (50 mM, final concentration) in phosphate buffer were added to the EPR cell. EPR spectra were obtained at X-band at room temperature on a Bruker ECS 106 EPR spectrometer equipped with rectangular cavity. Typical settings of spectrometer conditions were: microwave frequency, 9.82 GHz; microwave power, 20 mW; modulation amplitude,

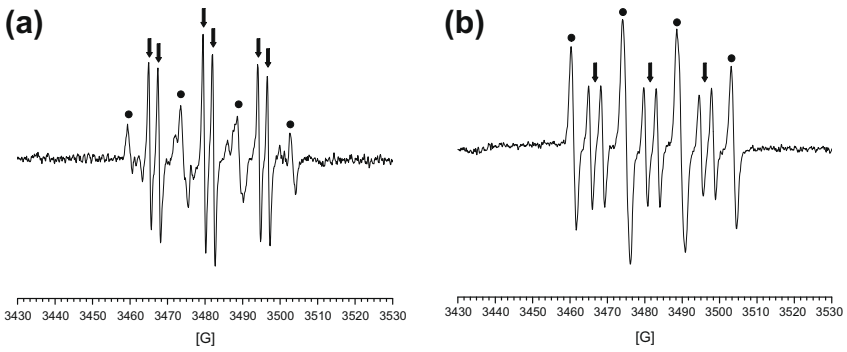


Figure 8. EPR spectra obtained after UV-photolysis in aqueous solution of hydrogen peroxide, DMPO-·OH spin adduct was marked with dots and **FxBN**-·OH spin adduct (a) or nitrone **7**-·OH spin adduct (b) were marked with arrows.

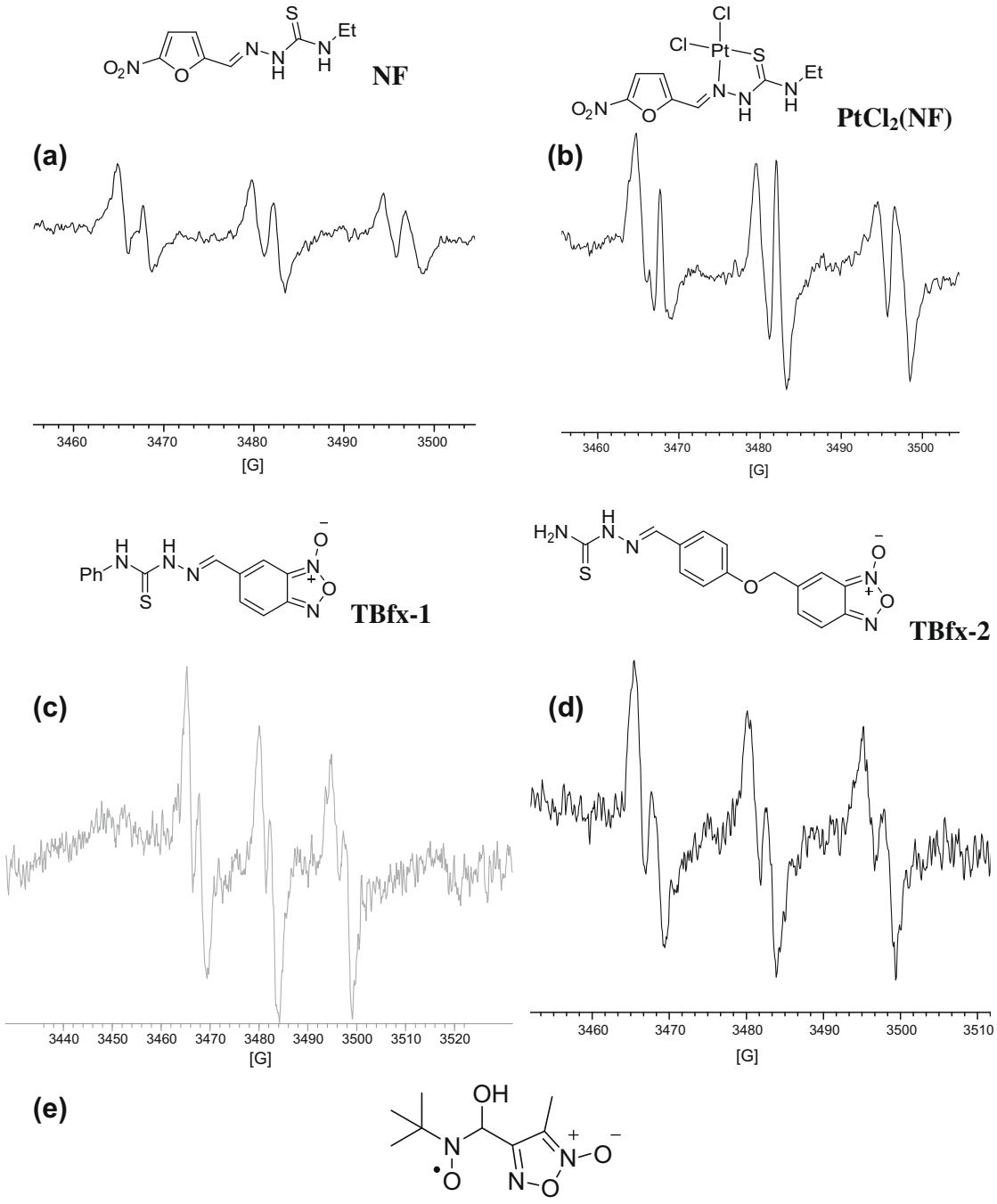


Figure 9. (a) Spectrum obtained when **FxBN** traps exclusively hydroxyl free radical produced by NF in presence of *T. cruzi* microsomal fraction. (b) Spectrum obtained with $PtCl_2$(NF) in the same biological system. Spectra (a) and (b) were presented in the same scale and performed in the same conditions. (c) Spectrum obtained when **FxBN** traps exclusively hydroxyl free radical produced by TBfx-1 in presence of *T. cruzi* microsomal fraction. (d) Spectrum obtained with TBfx-2 in the same biological system. Spectra (c) and (d) were presented in the same scale and performed in the same conditions. (e) Structure of the expected **FxBn**-˙OH spin adduct.

0.2 G; scan rate, 1.25 G/s; time constant, 0.5 s; number of scans 15. The EPR spectra were simulated using the EasySpin software.[47]

4.2.1. Estimation of rate constant and half-life for FxBN–hydroxyl adduct

Decay constant of **FxBN**–hydroxyl adduct was measured as follows: an aqueous solution, containing a mixture of phosphate buffer (pH 7.4) and 1% hydrogen peroxide is UV-illuminated. After 1 min of illumination the decay of the spin adduct was followed by monitoring the decrease of an appropriate EPR line.[34,48] Pseudo first-order decay of the spin adduct was measured by repeatedly recording the EPR signal intensity. Although the decay should follow a second order kinetics under these experimental conditions (were the spin traps concentration is present in large excess), it can be treated as pseudo first-order, that is, the half-life is not a function of component concentration. Also, the decay rate was calculated using the double integral of the EPR spectra, thus it may differ from the rate calculated from the concentration. Relative spin trapping rate for hydroxyl radical was determined using a competitive-trapping method,[48] where two spin traps are mixed and the EPR signal intensities are compared using the double integral of the recorded spectra, in this case DMPO and **FxBN**, and

DMPO and nitrone **7**. The general reaction scheme for spin trapping in this case of the hydroxyl radical in the presence of the two spin traps compounds is detailed as follows:

$$H_2O_2 \xrightarrow{hv} 2\cdot OH$$

$$DMPO + \cdot OH \xrightarrow{k_1} \cdot DMPO - OH$$

$$Nitrone(x) + \cdot OH \xrightarrow{k_2} \cdot Nitrone(x) - OH$$

The ratio of the assumed first order rates on the formation of the two spin adducts can be expressed as follows:

$$\frac{I_{DMPO}}{I_{Nitrone(x)}} = \frac{k_{1DMPO}}{k_{2Nitrone(x)}}$$

In this treatment we seek to relate the intensities of the signal found in the EPR spectra with the trapping rate constant relative to DMPO, which has been previously determined.

4.2.2. Estimation of half-life for FxBN–superoxide adduct

The half-life of **FxBN**–superoxide adduct was determined by monitoring the decrease in the first line of the EPR spectrum as a function of time. In a typical experiment, the reaction mixture contained **FxBN** (50 mM), xanthine (400 μM) in potassium phosphate buffer (100 mM, pH 7.4, containing 1 mM DTPA) and sufficient xanthine oxidase, generating $O_2^{-}\cdot$ at 1 μM/min, for 8 min, and then SOD (30 U/mL) was added. The reaction mixture was immediately transferred to an EPR flat quartz cell and introduced into the cavity of the EPR spectrometer. EPR spectra were continually recorded for 60 min.

4.3. Free radicals-production studies into *T. cruzi* microsomes

The spin trap capability of the new furoxanyl **FxBN** was assessed in the *T. cruzi* microsomes using 4-ethyl-1-(5-nitrofuran-2-ylmethylene)thiosemicarbazide (NF),[41] the corresponding platinum complex, for example, $PtCl_2$(NF),[42] N^4-phenyl benzofuroxan-5-carboxaldehyde thiosemicarbazone (TBfx-1) and 4-(benzofuroxan-5-ylmethyloxy)benzaldehyde thiosemicarbazone (TBfx-2),[6] as free radical producer agents. EPR spectra of each drug (1.0 mM, final concentration) was produced using the microsomal fraction (4 mg protein/mL) obtained from *T. cruzi* (Dm28c strain), in a reaction milieu containing 1 mM NADPH, 1 mM EDTA and 100 mM of **FxBN**, in 20 mM phosphate buffer, pH 7.4. The mixture was transferred to a 50 μL capillary. EPR 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.[49]

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