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ESR and electrochemical study of 1,2-disubstituted 5-nitroindazolin-3-ones and 2-substituted 3-alkoxy-5-nitro-2*H*-indazoles: Reactivity and free radical production capacity in the presence of biological systems

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ABSTRACT

Two families of 5-nitroindazole derivatives were electrochemically studied in an aprotic solvent using cyclic voltammetry (CV) technique. The produced nitro-anion radical species were characterized using electron spin resonance spectroscopy (ESR). Also, we examined the interaction between the radical species generated from nitroindazole derivatives and glutathione (GSH). Moreover, the capacity of intra-parasite and intramammals-free radical production, through ESR spectroscopy, was performed.

1. Introduction

Chagas disease (CD) is a major endemic parasitosis in Latin America [1] and is caused by *Trypanosoma cruzi* that is a protozoan parasite. This infection affects 16–18 million people mostly in rural regions of Latin America, and belongs to the group of neglected diseases as defined by the World Health Organization [2]. The parasite life cycle involves an extracellular, proliferative stage (*epimastigote*) that resides in the insect vector. Two forms occur in the mammalian host, a nonproliferating, infective form (*trypomastigote*) and an intracellular, proliferative form (*amastigote*) [3]. Congenital Chagas disease (CCD) has emerged as a public health problem due to the increasing control of the transmission of *T. cruzi* mediated by vector, blood transfusion and organ transplant [4]. Due to migration movements from endemic areas to vector-free suburban and urban centers, CCD is becoming increasingly responsible for the urbanization of CD [5].

Only two drugs, nifurtimox and benznidazole, are currently available to treat this disease but these are unsatisfactory given their toxic side effects and limited efficacy in the chronic phase of the infection. With no immediate prospect of a vaccine, the search for parasite-specific traits exploitable in terms of new chemotherapies is an urgent priority [2].

The mode of action of nifurtimox has been proposed to be due to the generation of reactive oxygen species [6–9]. In 1999, it has been shown that nifurtimox and benznidazole, the other drug used for CD, lower the intracellular thiol level of the parasite. Reduction of the nitroaromatic compounds, for instance to the nitroso derivatives, with the subsequent conjugation to thiols has been proposed as a mechanism [10].

While in most eukaryotic organisms the glutathione (GSH)/GR and thioredoxin (Trx)/TrxR systems maintain the intracellular thiol redox homeostasis, trypanosomatids possess a redox metabolism that is based on the low molecular mass dithiol trypanothione [bis(glutathionyl)spermidine; $T(SH)_2$] and trypanothione reductase (TR)—which keeps it in the reduced form [11,12]. $T(SH)_2$ is the central molecule for the detoxification systems of trypanosomatids [13].

The absence of the trypanothione system in mammals, the lack of a functional redundancy within the parasite thiol system together with the sensitivity of trypanosomes against oxidative stress, render the components of this metabolism attractive drug target molecules [14].

Non-selective bioreduction of the mentioned trypanocidal drugs could be the reason of its toxic effects in the mammalian host. The search for new compounds able to generate oxidative stress in *T. cruzi* through selective reduction by oxidoreductases unique in the parasite is an attractive target in the chemotherapy of CD [15].

The ability to produce free radical species capable of inducing a cascade of reduced materials, which are toxic towards the par-

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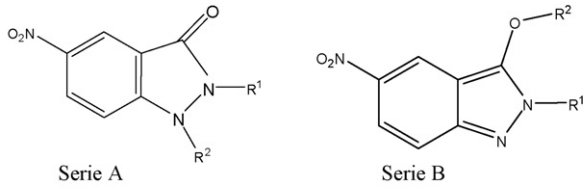

Derivatives	Serie	R^1	R^2	Derivatives	Serie	R^1	R^2
1	A	Bn	Me	**10**	B	Bn	Me
2	A	Bn	Pr	**11**	B	Bn	i-Pr
3	A	Ph	Me	**12**	B	Bu	Bu
4	A	Ph	i-Pr	**13**	B	Bn	Bn
5	A	Me	Me	**14**	B	Me	Bn
6	A	Me	Pr	**15**	B	Ph	i-Pr
7	A	Me	i-Pr	**16**	B	Bn	Pen
8	A	Me	Bn	**17**	B	Bn	Pr
9	B	Me	Me	**18**	B	Me	Pr

Fig. 1. Structure of 1,2-disubstituted 5-nitroindazolin-3-ones (series A) and 2-substituted 3-alkoxy-5-nitro-2*H*-indazole derivatives (series B).

asite, has been the proposed mechanism of action of many nitro compounds. These reduced intermediates can act as toxic agents against protozoa. For 5-nitroindazoles, QSAR studies demonstrate the importance of the volume for the trichomonacidal activity. Besides, for the anti-*T. cruzi* activity the significance of the lipophilic properties was observed. In this sense, it is necessary to investigate the mechanism of action of these compounds and to evaluate their interaction and radical production capacity in the presence of parasitic and mammals biological systems [16].

Many of the compounds showing antiprotozoal activity bear a nitro group in their heterocyclic structure. Thus, 2-benzyl-1-methylindazolinone **1** (see Fig. 1), presented appreciable activity and gave a very interesting result by showing inhibition percentages (%AE) higher than 80% at 100, 10, and 1 μg/mL against *T. cruzi* epimastigotes. However, the isomeric 1-benzyl-2-methylindazolinone **9** presented a similar %AE value (84%) at 100 μg/mL, but the activity was practically lost at lower concentrations (%AE of 23% and 16% at 10 and 1 μg/mL, respectively). On these bases, in order to explore the influence on the antitrypanosomal activity of different substituents at positions 1 and 2 of indazole ring, it was decided to prepare and test some anologues of compounds **1** and **9**, *i.e.*, 1,2-disubstituted 5-nitroindazolinones such as **2**–**8** and **10** (Fig. 1, series A) carrying benzyl, methyl or phenyl moieties at position 2, and different alkyl chains (benzyl, methyl, propyl or isopropyl) at position 1 [17].

In this paper two families of 5-nitroindazole derivatives, *i.e.*, the mentioned 1,2-disubstituted indazolinones **1**–**8** (Fig. 1, series A) as well as 2-substituted 3-alkoxy-2*H*-indazoles **9**–**18** (Fig. 1, series B), arising from the alkylation reactions leading to **1**–**8** (see below), have been electrochemically studied in aprotic solvent using cyclic voltammetry (CV) technique. The nitro-anion radical species were characterized by using electron spin resonance spectroscopy (ESR). Also, we examined the interaction between the radical species generated from nitroindazol derivatives and GSH. Moreover, the capacity of intraparasite and intramammals-free radical production, through ESR spectroscopy, was performed.

2. Experimental

2.1. Samples

1,2-disubstituted 5-nitroindazolin-3-ones (Fig. 1, series A) were prepared by treatment of the corresponding 2-substituted indazolin-3-ones with the required alkyl halides. It is generally assumed that this reaction only yields 1,2-disubstituted derivatives such as **1**–**8**. However, it has been found that in some cases, depending on the employed alkyl halides, considerable amounts of poorly known 2-substituted 3-alkoxy-2*H*-indazoles (**9**–**18**, Fig. 1, series B) are also obtained.

2.2. Cyclic voltammetry

Dimethylsulfoxide (DMSO) (spectroscopy grade) was obtained from Aldrich. Tetrabutylammonium perchlorate (TBAP), used as supporting electrolyte, was obtained from Fluka. CV was carried out by using a Metrohm 693VA instrument with a 694VA Stand convertor and a 693VA Processor, in DMSO (*ca.* 1.0 mmol L^{-1}), under a nitrogen atmosphere at room temperature, with TBAP (*ca.* 0.1 mol L^{-1}), using a three electrode cell. A hanging mercury drop electrode was used as the working electrode, a platinum wire as the auxiliary electrode and saturated calomel as the reference electrode.

2.3. ESR spectroscopy

ESR spectra were recorded in the X band (9.7 GHz) using a Bruker ECS 106 spectrometer with a rectangular cavity and 50 kHz field modulation. The hyperfine splitting constants were estimated to be accurate within 0.05 G. The anion radicals were generated by electrolytic reduction *in situ* under the same conditions of temperature, atmosphere, concentrations and with the same electrodes used in the CV experiment. The ESR spectra were simulated using the program WINEPR Simphonia 1.25 version.

2.4. Biological assays

The free radical production capacity of nitroindazolinone derivatives from A series was assessed on *T. cruzi*-microsomal fraction (4 mg protein/mL) by ESR using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) for spin trapping [18–21]. Nitroindazolinones (1 mM, final concentration) were dissolved in DMSO (spectroscopy grade) and the solution was added to a reaction medium containing 1 mM NADPH, 1 mM EDTA, and 100 mM DMPO, in 20 mM phosphate buffer (pH 7.4). The final mixture was transferred to a 50 μL capillary. All spectra were registered in the same scale after 15 scans.

The same methodologies were assessed on rats-microsomal fraction (4 mg protein/mL) biological system.

3. Results and discussion

3.1. Cyclic voltammetry

For all 5-nitroindazole derivatives (1 mM) we obtained typical voltammograms shown in Fig. 2. These voltammograms represent a one electron reversible transference process (peak I_{pc}/I_{pa}, around −1.0 V) corresponding to the generation of the nitro-anion free radical $RNO_2^{\bullet -}$ derived from 5-nitroindazoles.

From the study of the dependence of the cathodic peak current, I_{pc}, with the sweep rate, we obtained a linear dependence showing that the one electron reversible transference corresponds to diffusion controlled process without adsorption interference (Fig. 2, inset b). In addition, the current ratio, I_{pa}/I_{pc}, increases when the sweep rate increases up to 1.0 (Fig. 2 inset a). This result could indicate a typical variation of a pure electrochemical cuasi-reversible mechanism, showing values lower than 1.0 at low sweep rates and values around 1.0 at higher sweep rates. In this sense, the electrochemically generated nitro-anion radical could undergo two different decay paths, disproportionation or dimerization. Table 1 lists the values of voltammetric cathodic and anodic peaks for all the compounds studied and nifurtimox (Nfx). All derivatives exhibited more negative potential values than Nfx (−0.90 V) indicating a lesser capacity to be reduced. Therefore we use the theoretical

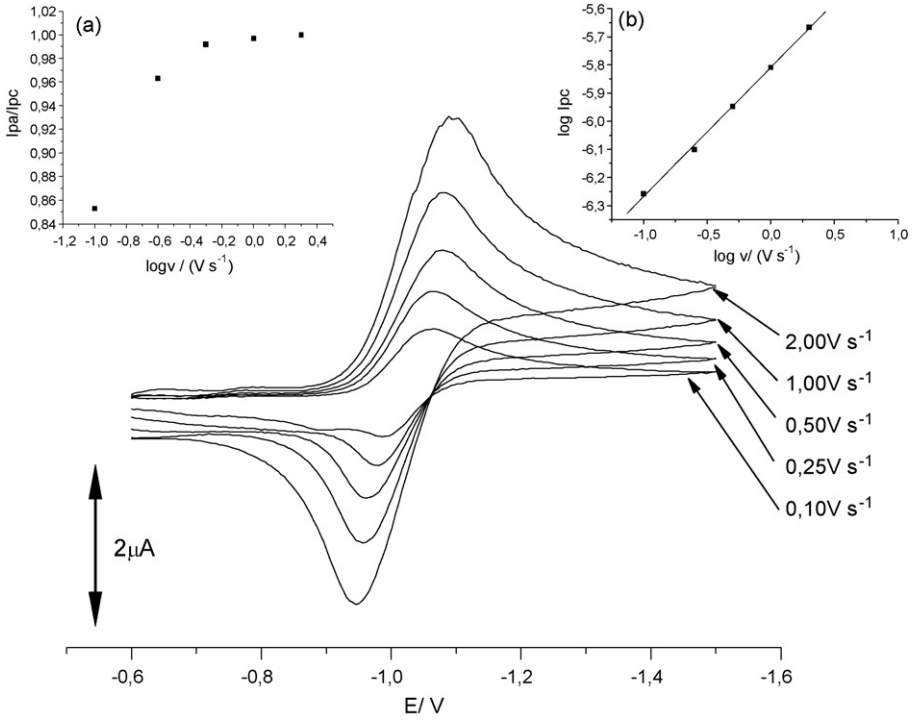

Fig. 2. Cyclic voltammograms of the isolated $RNO_2/RNO_2^{\bullet-}$ couple of nitroindazolinone **7** (1.0 mM) derivative in aprotic medium (DMSO + 0.1 M TBAP) and at different sweep rates (between 0.1 and 2.0 V s^{-1}). Insets: (a) current ratio vs. log (sweep rate); (b) cathodic peak current vs. log (sweep rate).

approach of Olmstead et al. for a dimerization or disproportionation coupled reaction [22], we have calculated the second-order rate constant (k_2), obtaining values between 217 and 226 M^{-1} s^{-1} for all the nitroindazole derivatives, these lower values indicate the stability of the nitro-anion radical species in aprotic media.

3.2. Reactivity of the nitro-anion radical electrochemically generated from 5-nitroindazole derivatives with GSH

We studied the reactivity of GSH with the nitro-anion radical of nitroindazole derivatives by CV by adding increasing 0.1 M GSH aqueous amounts (in buffer phosphate pH 7.4) to the media. Fig. 3 shows the typical CV behavior of nitroindazole derivatives in DMSO solutions in the absence and in the presence of GSH. This couple changes when GSH was added, as indicated by arrows. It can be observed that the cathodic peak increases significantly, and the absence of the return oxidation step is observed. GSH signals did not interfere the corresponding nitro-anion radical detection at the studied concentrations, only we can see part of the redox couple of this thiol by a new anodic peak that appears when increases the GSH amounts. These results indicated that the electrochemically obtained nitro-anion radical $RNO_2^{\bullet-}$ is immediately re-oxidized to the original material by the action of GSH. The species responsible for redox cycling, could be the GS• radical, produced via the 1 electron oxidation of GSH [23]. This radical species is the oxi-

Table 1
Characteristic CV parameters in DMSO vs. saturated calomel electrode (sweep rate 2 V s^{-1}).

Derivative	E_{pc} (V)[a]	E_{pa} (V)[b]	ΔE (V)	$\Delta E_{1/2}$ (V)[c]	I_{pa}/I_{pc}
1	−1.07	−0.96	−0.11	−1.02	1.02
2	−1.07	−0.97	−0.10	−1.02	1.02
3	−1.04	−0.93	−0.11	−0.98	0.99
4	−1.04	−0.90	−0.14	−0.97	1.01
5	−1.10	−0.95	−0.15	−1.03	0.97
6	−1.10	−0.98	−0.12	−1.04	1.02
7	−1.10	−0.95	−0.15	−1.02	1.00
8	−1.10	−0.92	−0.18	−1.01	1.00
9	−1.22	−0.99	0.22	−1.11	1.02
10	−1.20	−1.00	0.19	−1.09	1.03
11	−1.16	−1.00	0.16	−1.08	0.99
12	−1.17	−1.02	0.15	−1.09	1.01
13	−1.20	−1.02	0.18	−1.11	0.84
14	−1.18	−1.02	0.16	−1.11	0.83
15	−1.13	−0.96	0.17	−1.05	0.97
16	−1.17	−0.01	0.16	−1.09	1.00
17	−1.17	−1.00	0.16	−1.09	1.00
18	−1.19	−1.01	0.17	−1.10	1.01
Nifurtimox	−0.90	−0.18	−0.09	−0.86	0.91

[a] E_{pc} = cathodic peak potential.
[b] E_{pa} = anodic peak potential.
[c] $\Delta E_{1/2} = (E_{pc} + E_{pa})/2$.

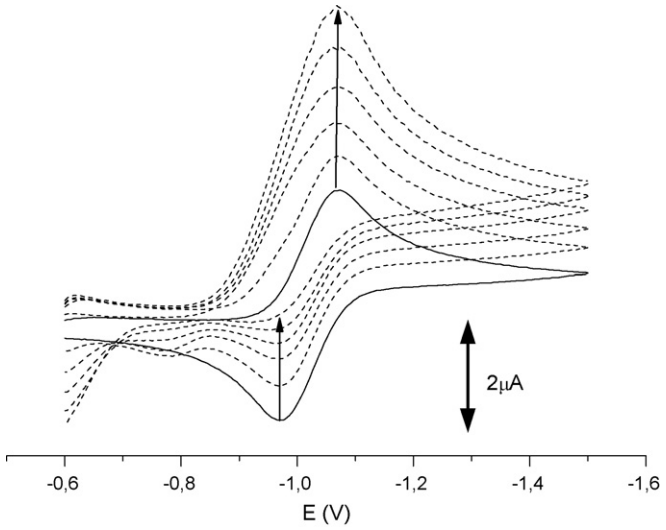

Fig. 3. CV of nitroindazolinone **2** (1.0 mM) in DMSO (0.1 M TBAP) for different amounts of GSH (1.0–5.0 mM), sweep rates 2.0 V s^{-1}.

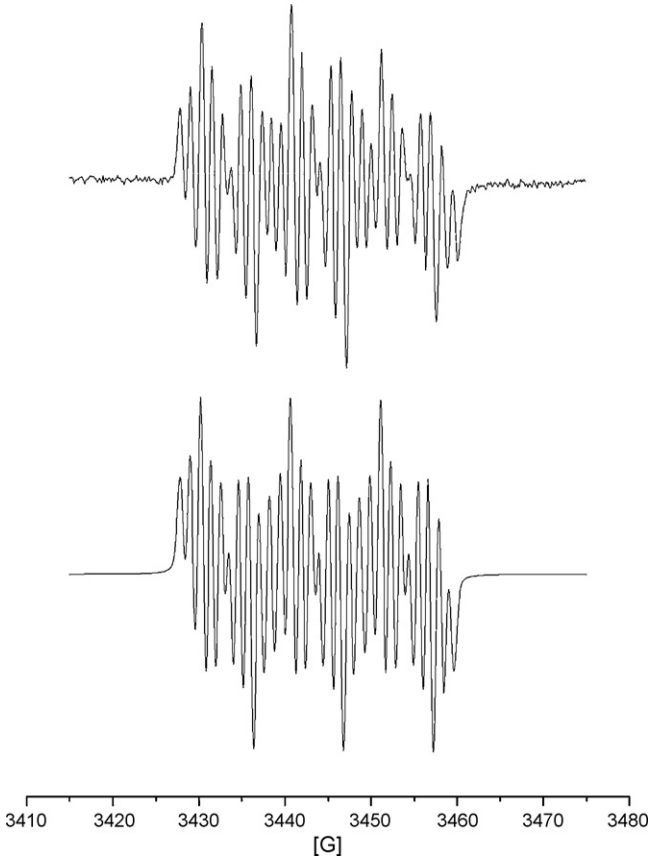

Fig. 4. ESR experimental spectrum of the anion radical of compound **1** in DMSO (up) and computer simulation of the same spectrum (down). Spectrometer conditions: microwave frequency, 9.70 GHz; microwave power, 20 mW; modulation amplitude, 0.87 G; receiver gain, 30 db. Spectrum was simulated using the following parameters: line width = 0.75 G, Lorentzian/Gaussian ratio = 0.6 and hyperfine constants are included in Table 2.

dizing agent for the $RNO_2^{\bullet -}$. Taking in mind that high thiol levels are present in biological medium, this kind of process could take place in the parasite explaining the observed biological activity for 5-nitroindazole derivatives against *T. cruzi*.

3.3. Electron spin resonance

The nitroindazole derivatives from series A and B were performed in ESR by electro reduction of these compounds *in situ*, by applying the potential corresponding to peak I_{pc} obtained from cyclic voltammetry experiments in DMSO. The simulation of the spectra was made by using hyperfine coupling constants (hfccs) obtained experimentally, modifying the line width, modulation amplitude and Lorentzian/Gaussian component until the resulting spectra reached the greatest similarity with the experimental ones. We obtained the same hyperfine pattern for the series A (Fig. 4), and was simulated in terms of three triplets assigned to the nitrogen nuclei of the nitro group, N-1 and N-2, and three doublets assigned to nuclei H-4, H-6 and H-7. This hyperfine pattern indicates that the nitro-anion radical species have a dislocated electron for all the nitroindazolinone ring including the N-1 and N-2, independent of their substituents. The obtained hfccs are shown in Table 2. On the other hand, for the series B, the hyperfine pattern (Fig. 5) shows a minor interaction between the electron and the N-2, with two triplets assigned to the nitrogen nuclei of the nitro group and N-1 and three doublets assigned to nuclei H-4, H-6 and H-7, resulting in a different hyperfine patterns respect to series A.

Table 2
Hyperfine coupling constants and *g* value of the simulated nitroindazole derivatives free radical spectrum.

Series	N_{NO_2}	N-1	N-2	H-4	H-6	H-7	*g*-value
A	10.44	1.18	0.20	4.35	2.40	1.00	2.0125
B	12.5	0.90	–	5.82	099	0.97	2.0128

3.4. Free radical production capacity of the nitroindazolinones in biological systems

In order to study the nitroindazolinones intraparasite-free-radical producer ability we selected the series A compounds. For this purpose we used ESR spectroscopy and spin-trapping tools [24]. According to these experiments nitroindazolinones were able to produce free radicals in the presence of *T. cruzi*-microsomal fraction. For example, we selected compounds **1** and **8** due to their *T. cruzi* growth inhibition capability and their less inhibition properties, respectively [16]. The ESR spectra for compound **1** showed a hyperfine pattern consistent with the nitro-anion free radical DMPO-trapped (Fig. 6). Additionally, showed the characteristic signals indicated by arrows in Fig. 6, of a decomposition by the oxidation of DMPO in presence of oxygen, called DMPO-X (5,5-dimethyl-2-oxo-pyrroline-1-oxyl) by Bilski et al. [25]. This could confirm that this compound was able to produce oxidative stress into the parasite. In the case of the less potent derivative **8**, the presence of radicals is observed but in a minor intensity. This is confirmed from the spectrums of Fig. 6.

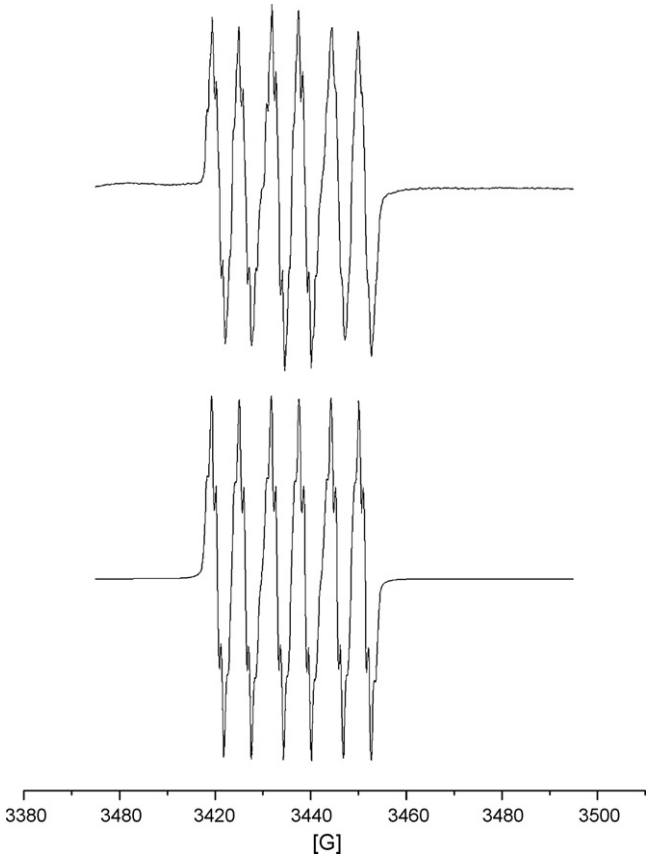

Fig. 5. ESR experimental spectrum of the anion radical of compound **13** in DMSO (up) and computer simulation of the same spectrum (down). Spectrometer conditions: microwave frequency, 9.68 GHz; microwave power, 20 mW; modulation amplitude, 0.87 G; receiver gain, 30 db. Spectrum was simulated using the following parameters: line width = 0.91 G, Lorentzian/Gaussian ratio = 0.6 and hyperfine constants are included in Table 2.

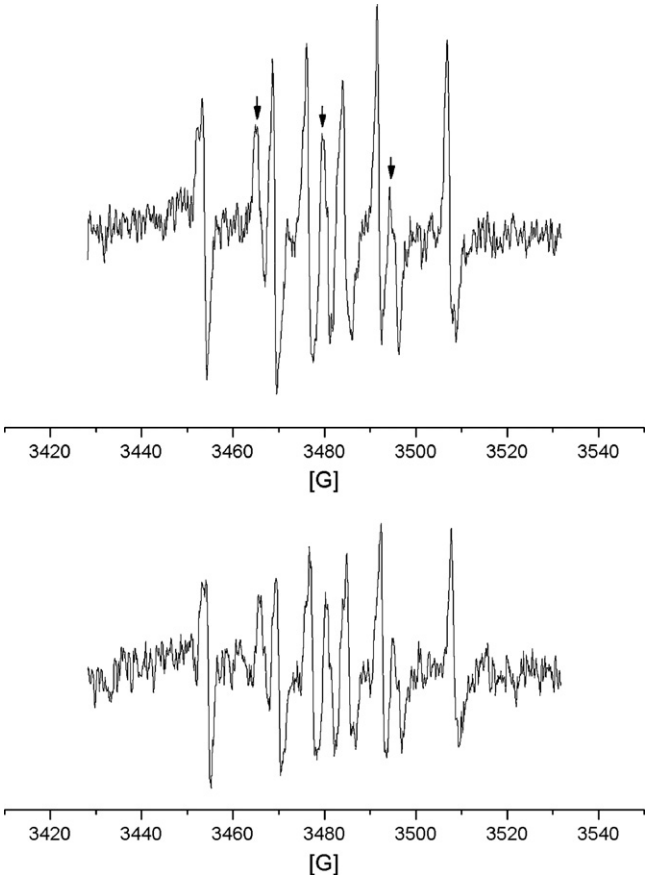

Fig. 6. ESR spectra DMPO-nitro-anion radical spin adduct obtained from nitroindazolinones **1** (up) and **8** (down) 1 mM final concentration, with *T. cruzi*-microsomal fraction (4 mg protein/mL). The arrows in the ESR spectra indicate the DMPO-X signal.

An interesting behavior is presented from these nitroindazolinones in the presence of rats-microsomal system. The spectrum in Fig. 7 presents lesser intensities than in the presence of *T. cruzi*-microsomal fraction biological system, only in the molecules with an aliphatic group in N-1 position. In the case of the rest of the samples, these intensities are practically the same that we obtained for the nitroindazolinones in the presence of *T. cruzi*-microsomal fraction.

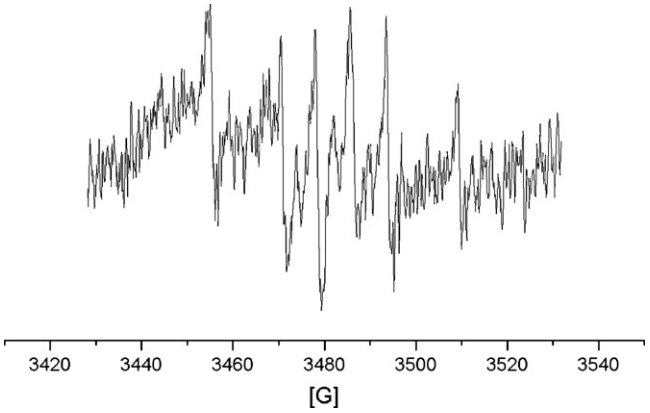

Fig. 7. ESR spectrum of DMPO-nitro-anion radical spin adduct obtained with rats-microsomal fraction (4 mg protein/mL) and nitroindazolinone **1** (1 mM final concentration).

4. Conclusions

The CV study of the studied nitroindazole derivatives shows a reduction process corresponding to the generation of the nitro-anion radical species and a lesser capacity than nifurtimox to be reduced. This mechanism is independent of the substituent groups in the different positions on the molecule, as was found by the ESR study that evidenced the presence of the electron dislocation in the nitroindazole ring for all the samples through a very well defined spectrum with different hyperfine patterns for the A and B series. GSH was capable of acting as an oxidizing agent for the 5-nitroindazole derivatives, regenerating the starting material from the nitro-anion radical. The oxidizing effect of GSH is supported by the parallel decrease of the anodic peak current and the increase of the cathodic peak in the CV, corresponding to the nitro-anion radical wave from uncharged species with the addition of GSH. On the other hand, we used ESR spectroscopy and trap the free radicals produced by the nitroindazolinones of the series A in the presence of *T. cruzi*-microsomal and a mammal-microsomal fraction. These results show that the molecules, with aliphatic substituents in position N-1, present a higher free radical production capacity, in the presence of *T. cruzi*, than the nitroindazolinones with aromatic substituent in the same position. We observed a decrease in the spectrum intensity in the presence of rat-microsomes, indicating a minor free radical production in the presence of this biological system.

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