Electrochemical and microsomal production of free radicals from 1,2,5-oxadiazole N-oxide as potential antiprotozoal drugs

C. Olea-Azar [a,*], Carolina Rigol [a], Fernando Mendizábal [b], R. Briones [a], Hugo Cerecetto [c], Rossana Di Maio [c], Mariela Risso [c], Mercedes González [c], Williams Porcal [c]

[a] *Departamento de Química Inorgánica y Analítica, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Olivos 1007, Cassilla 233, Santiago, Chile*

[b] *Departamento de Química, Facultad de Ciencias Universidad de Chile, Olivos 1007, Cassilla 233, Santiago, Chile*

[c] *Departamento de Química Orgánica, Facultad de Química and Facultad de Ciencias, Universidad de la República, Uruguay*

Abstract

The electron spin resonance (ESR) spectra of free radicals obtained by electrolytic or microsomal reduction of several potential antiprotozoal 1,2,5-oxadiazoles were characterized and analyzed. Ab initio molecular orbital calculations were performed to obtain the optimized geometries and the theoretical hyperfine constant was carried out using ZINDO semiempirical methodology. Density functional theory was used to rationalize the reduction potentials of these compounds.

Keywords: ADF; Cyclic voltammetry; ESR; Microsome; N-oxide derivates anion radical; *T. cruzi*

1. Introduction

Trypanosoma cruzi is the etiologic agent of Chagas' disease. The trypanosiomases and leishmaniases are mainly Third World diseases and Chagas' disease affects ≈24 million people from Southern California to Argentina and Chile [1,2]. The current chemotherapy against Chagas' disease is still inadequate. Many experiments carried out on the only two drugs commonly used to treat Chagas' disease suggest that their intracellular reduction followed by redox cycling yielding $O_2^{-\cdot}$ and eventually $OH^{\bullet}$, may be their major mode of action against *T. cruzi* [3–5].

We have previously reported studies on antiprotozoal 5-nitrofurfural and 5-nitrothiophene-2-carboxaldehyde derivatives. These compounds were shown to generate nitro anion radicals, that were characterized using ESR spectroscopy [6,7]. Similar to the nitro pharmacophore of antitrypa-

* Corresponding author. Tel.: +56-2-678-2846; fax: +56-2-737-0567

E-mail address: colea@uchile.cl (C. Olea-Azar).

nosomal drugs, the N-oxide moiety has proved to be responsible for the biological activity of different drug families (with antitumor or antibacterial activities) through the production of free radical species [8,9].

We recently reported studies on the 1,2,5-oxadiazole N-oxide family in order to determine their antitrypanosomal activities, tested in vitro against the epimastigote form of *T. cruzi*. Moreover, we have shown some ESR spectra that prove the facile electronation of the N-oxide moiety. Beside, these new structures were based on the conjunction of N-oxide systems and the semicarbazide moieties ('spermidine-mmetic') [10].

In the present work, we have characterized the free radical species derived from this N-oxide family (Fig. 1), generated either by electrolytic or microsomal reduction, using ESR spectroscopy.

To estimate the theoretical hyperfine splitting constants, ZINDO semiempirical methodology were carried out. The geometry of each compound in both spin paired and free radicals species was fully optimized at ab initio STO 3-21G level.

2. Experimental section and theoretical methods

2.1. Samples

The N-oxides were synthesized according to methods described earlier [10].

2.2. Cyclic voltammetry

DMSO and DMF (spectroscopy grade) were obtained from Aldrich. Tetrabutylammonium perchlorate (TBAP) used as supporting electrolyte was obtained from Fluka. Cyclic voltammetry was carried out using a Weenking POS 88 instrument with a Kipp Zenen BD93 recorder, in DMSO or DMF (ca. 1.0×10^{-3} mol dm^{-3}), under a nitrogen atmosphere, with TBAP (ca. 0.1 mol dm^{-3}), using three-electrode cells. A mercury-dropping electrode was used as the working electrode, a platinum wire as the auxiliary electrode and saturated calomel as the reference electrode. The N-oxide radicals were generated by electrolytic reduction in situ at room temperature.

2.3. ESR spectroscopy

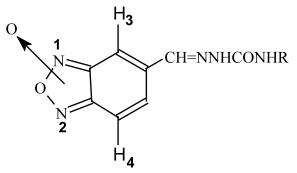
ESR spectra were recorded in the X band (9.85 GHz) using a Bruker ECS 106 spectrometer with a rectangular cavity and 50 kHz field modulation. The hyperfine splitting constants were estimated to be accurate within 0.05 G.

2.4. Microsomal experiment

The experiments were run in Tris buffer (150 mM KCl, 50 mM Tris–HCl, pH 7.36 at 25 °C) treated with Chelex ion exchange resin before use to remove adventitiously present metal ions. NADPH, NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Sigma. All chemicals were dissolved in the buffer (adding minimal quantities of DMSO, as necessary, for the N-oxide derivatives). Incubations consisted of microsomes homogenized in buffer (typically 3 mg of protein/ml), Anthracenetrione derivatives (5.0 mM) glucose-6-phophate (10 mM) with glucose-6-phophate dehydrogenase as an NADPH-generated system and NADPH (1 mM). The rat liver microsomes were prepared by published methods and kept frozen at −70 °C until used. Buffer was added if necessary to maintain a constant total volume for all experiments. In all cases, the NADPH was added immediately before the EPR spectrum was recorded.

2.5. Theoretical calculations

Full geometry optimizations of the N-oxides in spin-paired and free radical forms were carried out



Ia R= butyl
Ib R= hexyl
Ic R= 3-(dimethylamino)propyl
Id R= 3-(diethylamino)propyl

Fig. 1. Chemical structure of the N-oxides derivates.

Table 1
Cyclic voltammetric parameters of N-oxide derivatives versus saturated calomel electrode

N-oxide	E_{pc1}/V	E_{pa1}/V	ΔE/V	$E_{1/2}$/V	E_{pc2}/V	E_{pa2}/V
Ia						
DMSO	−1.07	−0.95	0.12	−1.01	−1.62	–
DMF	−1.06	−0.91	0.15	−0.99	1.60	–
Ib						
DMSO	−1.09	−0.96	0.13	−1.03	−1.65	–
DMF	−1.05	−0.91	0.14	−0.98	−1.62	–
Ic						
DMSO	−1.04	−0.92	0.12	−0.98	−1.61	–
DMF	−1.02	−0.90	0.12	−0.96	−1.59	–
Id						
DMSO	−1.05	−0.93	0.11	−0.99	−1.63	–
DMF	−1.04	−0.92	0.12	−0.98	−1.60	–
NIFURTIMOX						
DMSO	−0.91	−0.85	0.06	−0.88	−1.60	–
DMF	−0.89	−0.84	0.05	−0.87	−1.30	–

by STO 3-21G methods [11]. STO-3-21G calculations were carried out employing the open shell UHF option. The theoretical hyperfine constant were carried out using ZINDO semiempirical methodology. The electron affinities were calculated using ADF calculations. This approach solves the Kohn–Sham equations within the local density approximation (LDA) with gradient corrections for the exchange and correlation potentials. The ADF basis set used was a doublet-ξ plus polarization functions. The LDA exchange correlations suggested by Vosko [12] were used, as well as the nonlocal gradient corrections for exchanges proposed by Becke and the nonlocal correction for correlation proposed by Perdew [13].

3. Results and discussion

3.1. Cyclic voltammetry

Table 1 lists the values of the voltammetric peaks and the anodic and cathodic currents for the three compounds studied. These N-oxides displayed comparable voltammetric behavior, showing two well-defined reduction waves both in DMSO and in DMF.

The first wave corresponds to a quasireversible process. We have studied the stability of the species generated by changing the electrochemical conditions, i.e. the scan rate and the switching potential, while keeping the chemical conditions of the solution constant. Results show that as the scan rate increases, the Ipa/Ipc ratio increases, as is typical for a reversible chemical reaction following a reversible charge transfer [14]. On the other hand, the side chains assessed in this work did not modify the $E_{1/2}$, however it showed that the change from alkylamino chain to (*N*,*N*-dialkyl-amino)alkylamino group (spermidine-mimetic group) resulted in a significant decrease in the antitrypanosomal activity indicated that lipophilic or hridrofilic properties maybe related with the activities [10].

The second cathodic peak is irreversible in the whole range of sweep rates used (50–2000 mV s^{-1}). We can attribute this wave to the production of the deoxygenated derivative with hydroxyl ion release. A small amount of water should be present in the aprotic solvents in order to provide the proton necessary for the chemical reactions observed in the first couple, which are summarized in the mechanism shown in Figs. 2 and 3.

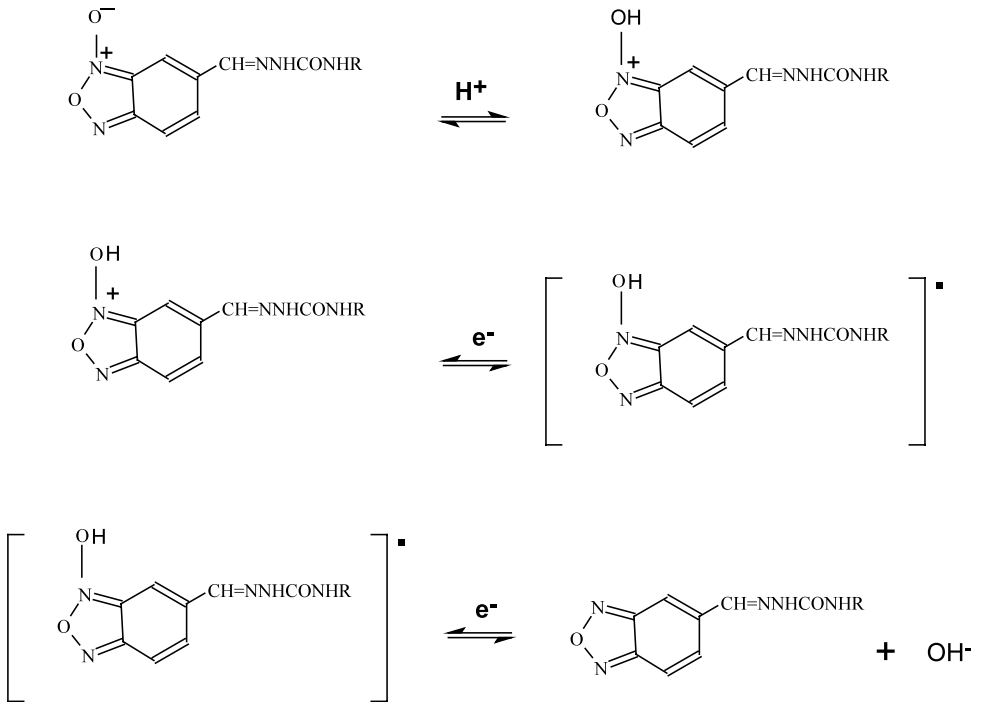


Fig. 2. Reduction mechanism proposed to N-oxides derivates.

3.2. Electron spin resonance

The N-oxide free radicals were prepared in situ by electrochemical reductions in DMSO, applying a potential corresponding to the first wave for the N-oxides, as obtained from the cyclic voltammetric experiments. The interpretation of the ESR spectra by means of a simulation process led to the determination of the coupling constants for all the magnetic nuclei, confirmed by theoretical calculations.

All ESR spectra of the N-oxides were analyzed and simulated in terms of a doublet arising from the hydrogen nuclei provided by the protonation involved in the mechanism described above, which gave a larger hyperfine constant than any others. Two triplets could be assigned to the two nitrogen atoms of the N-oxide and two doublets were accounted for by two aromatic ring hydrogen atoms. The hyperfine splitting constants are listed in Table 2.

The microsomal incubations of all compounds gave an ESR spectrum after a brief induction period of 1–2 min, which was required for the incubation to become anaerobic. Figs. 4 and 5 show the ESR spectra of compounds Ia and Ic and its corresponding simulation, respectively. The hyperfine splitting pattern of the biochemically generated free radicals was the same as that obtained by electrochemical reduction. Also, we

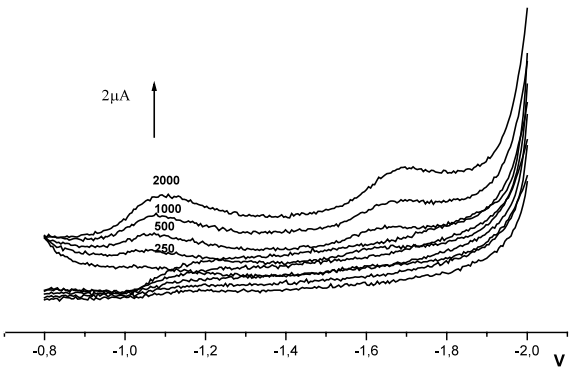


Fig. 3. Cyclic voltammetry of Ib in DMSO at different sweep rates.

Table 2
Experimental and theoretical hyperfine splittings (Gauss) and g values for the N-oxide anion radicals studied in DMSO

N-oxides	aH (protonation)	aN1	aN2	aH3	aH4	g Values
Ia						
EXP	7.25	4.05	2.90	1.15	0.97	2.0057
INDO	8.50	5.90	2.00	0.89	0.45	
Ib						
EXP	11.25	6.95	1.60	4.0	0.3	2.0060
INDO	13.00	7.50	1.20	2.9	0.09	
Ic						
EXP	7.25	4.05	2.90	1.30	0.97	2.0058
INDO	8.40	5.80	2.00	0.95	0.45	
Id						
EXP	10.75	6.75	3.65	1.04	0.5	2.0058
INDO	12.00	7.20	3.40	0.84	0.1	

produced the stable radicals of N-oxides Ib and Id generated by the microsomal system, which presented the same hyperfine splitting pattern as obtained by electrochemical generation.

3.3. Theoretical calculations

We fully optimized the geometries for the electron-paired and anion radical molecules at the ab initio STO-3-21G level. All free radical structures were optimized in their protonated forms, which showed little distortion with respect to the neutral analogues. The calculations on the unprotonated radical species for all compounds show a more homogenous spin distribution in the N-oxide aromatic ring, which does not agree with the experimental hyperfine constants. However,

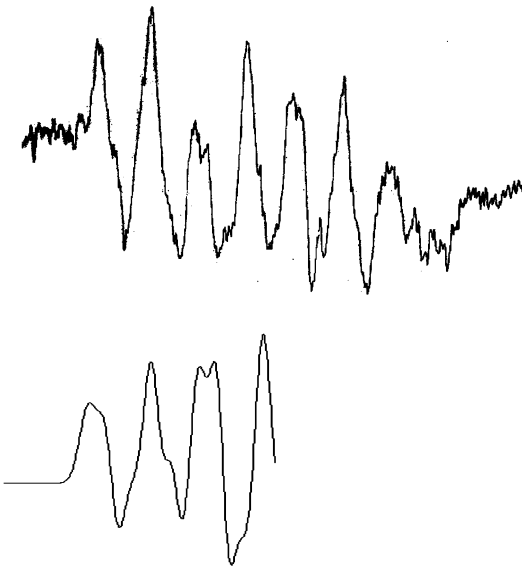


Fig. 4. (a) ESR spectrum of Ia N-oxide derivate generated by microsomal system; (b) computer simulation of the same spectrum.

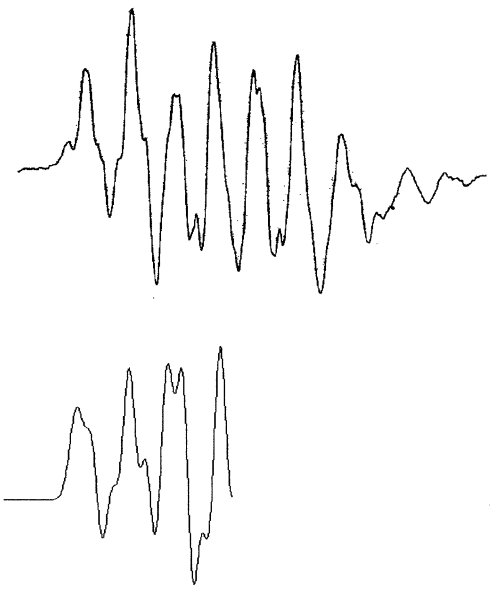


Fig. 5. (a) ESR spectrum of Ic N-oxide derivate generated by microsomal system; (b) computer simulation of the same spectrum.

Table 3
Electron affinities (eV) and reduction potentials (in DMSO)

N-oxide	LDA	LDA+NLC (Becke–Perdew)	$E_{1/2}$ (V)
Ia	−1.545	−1.559	−1.01
Ib	−1.313	−1.369	−1.03
Ic	−1.590	−1.618	−0.98
Id	−1.589	−1.609	−0.99

the theoretical hyperfine constants were in agreement with the experimental results when the protonation of the N-oxide was considered (Table 2).

In order to estimate the ability of the molecules to accept electrons, their electron affinities were calculated using ADF and comparisons were made with the formal reduction potentials of the anion radical forms (Table 3). The calculations show that the electron affinities of these N-oxides correlate well with the experimental values estimated from the reduction potentials.

4. Concluding remarks

All N-oxide studies showed similar $E_{1/2}$ to nifurtimox. Also, the side chains assessed in this work did not modify the $E_{1/2}$, an aspect that might be important for the selectivity of these compounds towards trypanothione reductase.

Stable free radicals generated using a microsomal system showed hyperfine coupling constants identical to those of the radicals obtained by electrochemical reduction. Also, the ESR spectra proved that the protonation of N-oxide group is protonated, as suggested by the reduction mechanism proposed on the basis of the cyclic voltammetric results. The theoretical calculations are in complete agreement with the experimental results and allowed the protonation step to be rationalized. Finally, the electron affinity calculated at the ADF level could be used to predict the reduction potential and estimate as a first approach if new drugs might be active against *T. cruzi*.

Acknowledgements

This research was supported by FONDECYT 1000834 Grant, CSIC No. 220, RELAQ (Mariela Risso and Carolina Rigol) and CEPEDEQ.

References

[1] Z. Brener, Pharmacol. Ther. 7 (1979) 71.
[2] J.C. Messender, L. Tinocco, J.D. Figueroa-Villar, E.M. Souza, R. Santa Rita, S.L. de Castro, Bioorg. Med. Chem. Lett. 5 (1995) 3079.
[3] B. Hazra, P. Sur, B. Sur, A. Banerjee, D.K. Roy, Planta Med. 51 (1984) 295.
[4] A. Morello, M. Pavani, J.A. Garbarino, M.C. Chamy, C. Frey, J. Mancilla, A. Guerrero, Y. Repetto, J. Ferreira, Comp. Biochem. Physiol. 112C (1995) 119.
[5] R. Docampo, S.N.J. Moreno, Rev. Infect. Dis. 6 (1984) 223.
[6] C. Olea-Azar, A. Atrua, R. di Maio, G. Seaone, H. Cerecetto, Spectrosc. Letts. 31 (1998) 849.
[7] C. Olea-Azar, A.-M. Atria, F. Mendizabal, R. di Maio, G. Seoane, H. Cerecetto, Spectrosc. Letts. 31 (1998) 99.
[8] A. Cahill, I.N. White, Biochem. Soc. Trans. 19 (1991) 1275.
[9] J.M. Brown, Redox activation of benzotriazine N-oxides: mechanisms and potential as anticancer drugs, In: Selective Activation of Drugs by Redox Processes, Plenum Press, Fermo, Italy, pp. 137–148.
[10] A. Monge, A. Lopez de Cearin, E. Díaz, R. di Maio, M. Gonzalez, S. Onetto, G. Seaone, H. Cerecetto, C. Olea-Azar, J. Med. Chem. 42 (1999) 1941.
[11] M.J. Risch, G.W. Trucks, H.B. Schlegel, P.N.W. Gill, B.G. Johnson, M.A. Robb, J.R. Cheeseman, K.T. Keith, G.A. Petersson, J.A. Montgomery, K. Raghavachari, M.A. Al-Laham, V.G. Zakrzewski, J.V. Ortiz, J.B. Foresman, J. Cioslowski, B.B. Stefanov, A. Nanayakkara, M. Challacombe, C.Y. Peng, P.Y. Ayala, W. Chen, M.W. Wong, J.L. Andres, E.S. Replogle, R. Gomperts, R.L. Martin, D.J. Fox, J.S. Binkley, D.J. Defrees, J. Baker, J.P. Stewart, M. Head-Gordon, C. Gonzalez, J.A. Pople, Gaussian '98, Rev. A.7, Inc., Pittsburgh, PA, 1998.
[12] S.H. Vosko, L. Wilk, J. Phys. Chem. B16 (1983) 3687.
[13] J. Perdew, Phys. Rev. B33 (1984) 8822.
[14] R. Nicholson, I. Shain, Anal. Chem. 36 (1964) 706.