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GENERATION OF RADICAL ANIONS OF NIFURTIMOX AND RELATED NITROFURAN COMPOUNDS BY ASCORBATE

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Nifurtimox analogues bearing triazol-4-yl, benzimidazol-1-yl, triazin-4-yl or related groups as counterpart of the (5-nitro-2-furfurylidene) amino group were reduced to their nitro anion radicals by ascorbate in anaerobic solutions at high pH. The ESR spectra of the radical anions showed hyperfine spin couplings restricted to the nitrofuran moiety. With these compounds, the spin density at the nitro group was greater than with nifurtimox, nitrofurazone and nitrofurantoin. At neutral pH, solutions containing ascorbate and nitrofuran derivatives consumed oxygen, the compounds bearing unsaturated nitrogen heterocycles being the most effective. Superoxide dismutase and catalase decreased the rate of oxygen consumption, thus demonstrating the production of superoxide and hydrogen peroxide, respectively. NMR spectra of the triazol-4-yl and triazin-4-yl nitrofuran derivatives showed a deshielding effect for the azomethine proton, which was undetectable with nifurtimox and nitrofurazone.

KEY WORDS: Nitrofurans, nitro anion radicals, nifurtimox analogues, ascorbate, superoxide.

Abbreviations and chemical terms used: NF, (5-nitro-2-furfurylidene)amino; nifurtimox, 3-methyl-4-[NF]-tetrahydro-4H-thiazine-1,1'-dioxide; NF-triazole, 4-[NF]-1,2,4-triazole; NF-pyrazole, 1-[NF]-pyrazole; NF-benzimidazole, 1-[NF]-benzimidazole; NF-triazole(I), 3,5-bis(methylthio)-4-[NF]-1,2,4-triazole; NF-triazole(II), 1-methyl-3-methyl-thio-4-[NF]-1,2,4-triazole-5-thione; NF-triazine, 3-thioxo-4-[NF]-6-methyl-1,2,4-triazin-5-one; nitrofurantoin, 1[[(5-nitro-2-furanyl) methylene]-amino]-2,4-imidazolidinedione; nitrofurazone, 5-nitro-2-furaldehyde semicarbazone; DMFA, N.N-dimethylformamide; DMFA-d_7, DCON$[CD_3]_2$; DETAPAC, diethylenetriaminepentaacetic acid; HFSC, hyperfine spin coupling constant.

INTRODUCTION

An important step in the metabolism of nitroheterocyclic compounds is their reduction to the corresponding radical anions which, under aerobic conditions, reduce oxygen to superoxide anion and hydrogen peroxide.[1-5] Ascorbate reduces the nitro group of nitrofuran compounds and Rao *et al.*[6] provided evidence for the formation of the nitro anion radical using nitrofurantoin, misonidazole and metronidazole. In the present study we have extended Rao *et al.*[6] observations to nifurtimox, nit-

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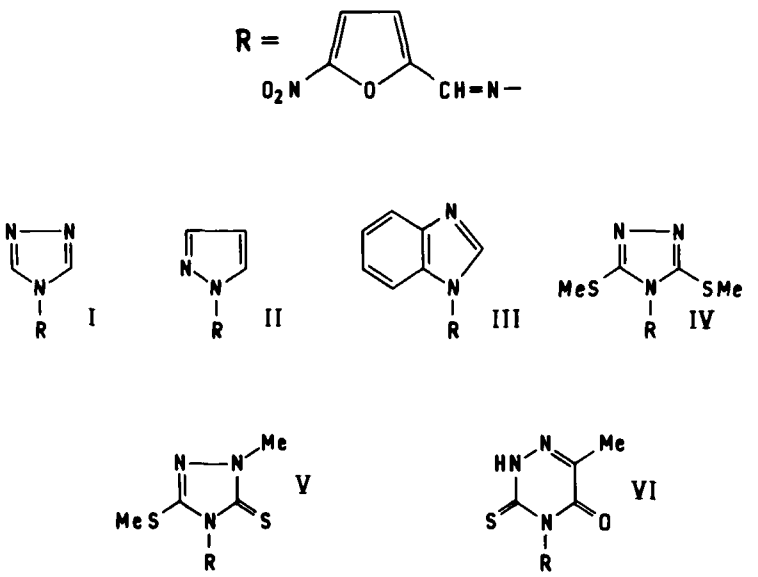

FIGURE 1 Structure of nifurtimox analogues. *R*, (5-nitro-2-furfurylidene) amino (NF in the text); I, 4*R*-1,2,4,triazole; II, 1*R*-pyrazole; III, 1*R*-benzimidazole; IV, 3,5-bis-(methylthio)-4*R*-1,2,4-triazole; V, 1-methyl-3-methylthio-4*R*-1,2,4-triazole-5-thione; VI; 3-thioxo-4*R*-6-methyl-1,2,4-triazin-5-one. Each compound consist of moieties A (structure *R*) and B (structures I-VI).

rofurazone and several analogues of nifurtimox bearing unsaturated five- or six-membered nitrogen heterocycles[7] (Figure 1). Nifurtimox is one of the most effective drugs used in the treatment of Chagas' disease (American trypanosomiasis)[4] and nifurtimox analogues include compounds with superior *in vitro* activity against *Trypanosoma cruzi*.[7] Furthermore, some of the new nifurtimox derivatives exert direct mutagenic activity upon *Salmonella typhimurium*[8] and are effective inhibitors of the enzyme glutathione reductase.[9]

MATERIALS AND METHODS

Sodium ascorbate, nitrofurazone, nitrofurantoin, 5-nitro-2-furoic acid, catalase (bovine liver), SOD (bovine erythrocytes), DMFA, DMFA-d_7, DETAPAC and Trizma were obtained from Sigma Chemical Co, St Louis, MO. Nifurtimox was a gift from Bayer A.G., Leverkusen, German Federal Republic, through the courtesy of Dr. A. Haberkorn; nifurtimox analogues were synthesized by Mester *et al.*[7] and supplied by Dr. R. Claramunt, Departamento de Química Orgánica, Facultad de Ciencias, U.N.E.D., 28040, Madrid, Spain. Other reagents were analytical grade.

ESR measurements were performed at room temperature (22–25°C) with a Bruker ER 200tt X-band ESR spectrometer equipped with TE_{102} cavity. A solution of 50 mM ascorbate in 0.1 N NaOH, in a 3 ml anaerobic cell, was bubbled with a stream of nitrogen for 5 min. At the same time, the nitrofuran solution in DMFA was also bubbled with nitrogen, as described. A sample of this solution was added to the anaerobic ascorbate solution and further bubbled with nitrogen for 1 min. Lastly, the reaction mixture was transferred anaerobically to the spectrometer cell previously

filled with nitrogen, the head space of the cell was flushed with nitrogen and the spectrum was recorded. Other experimental conditions were as described in the Results section. The g factors and $a(H_a)$ HFSC of the various radicals were determined against those of aqueous solutions of Fremy's salt ($(KSO_3)_2NO$). Computer simulation of the experimental spectra was carried out on a desktop computer using the correlation described.[10]

^{1}H-NMR spectra were recorded at 100.1 MHz on a Varian XL-100-15 spectrometer operating in the FT mode. Compounds were dissolved in DMFA-d_7 and analyzed at *ca.* 27°. Chemical shift values (δ) are indicated in ppm from tetramethylsilane (TMS) used as standard. Assays of oxygen consumption were carried out in 0.1 M Tris-HCl buffer, pH 7.5, 1.0 mM DETAPAC and 5.0 mM Na-ascorbate, with additions as described in Tables II and III. Measurements were performed with the Gilson Oxygraph, at 30°. Unless stated otherwise, the results presented are the average of duplicate measurements; the experimental values deviated from the mean value by less than 5%. Calculations were made with an Apple II Europlus PC.

RESULTS

Figure 2 shows the ESR spectrum of NF-triazole radical nitroanion. The hyperfine splittings of 12.4, 5.8 and 1.35 G indicated spin couplings at the nitro group nitrogen

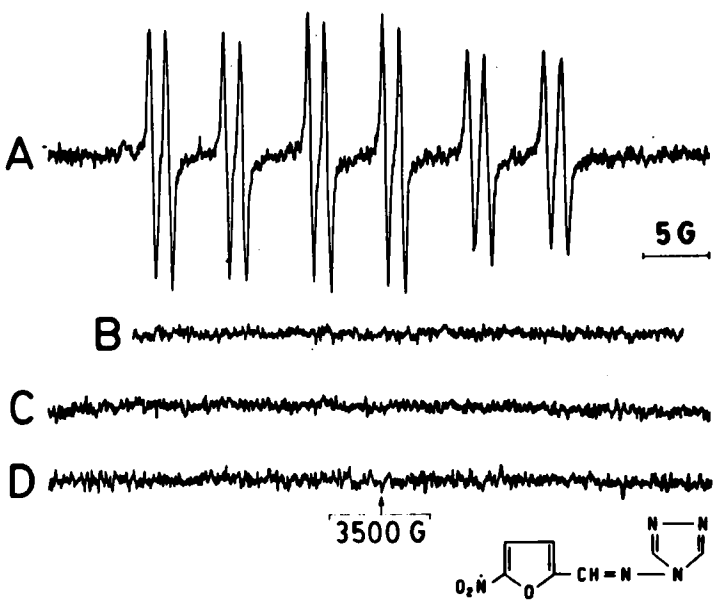

FIGURE 2 ESR spectra of radical anions produced in a system of 5.0 mM NF-triazole and 50 mM ascorbate in 0.1 N NaOH, under anaerobic conditions. A, complete system; B, sample with NF-triazole, without ascorbate; C, sample with NF-triazole in 100% (v/v) DMFA, without ascorbate; D, sample with ascorbate, without NF-triazole. Instrumental conditions: 21 mW microwave power; 100 kHz microwave modulation frequency, 0.20 Gpp modulation intensity; 0.20 G modulation amplitude; 0.5 s time constant; 6 G/min scan rate and 8 × 10^5 gain. Other experimental conditions were as described in the Materials and Methods section.

and also at protons at C-4 and C-3 of the furan ring. The radical signals were visualized during the first 10 min of incubation but thereafter disappeared. Spin coupling for the α-proton in the chain, the nitrogen in the –CH = N– group or other atoms in the non-nitrofuran moiety were undetectable. Omission of nitrofuran or ascorbate from the assay mixture led to a total loss of response. Figures 3 and 4 show the spectra of NF-triazole(I) and NF-benzimidazole nitroanion radicals, respectively. It may be seen that the corresponding hyperfine splittings were identical to those obtained with NF-triazole (Figure 2). With NF-triazole(I), measurements of signal amplitude at different nitrofuran concentrations demonstrated good correlation (Figure 3) and calculation of radical anion concentrations from 5 mM nitrofuran spectra (Figures 2–4) gave values in the 3.0–5.0 μM range. Simulation of the NF-benzimidazole ESR spectrum using the coupling constants obtained experimentally produced the results foreseen (Figure 4). With NF-triazine (Figure 5), the hyperfine splittings resembled those obtained with the triazole and benzimidazole derivatives (Figures 2–4), but the calculated radical concentration was relatively smaller (1.4 μM), thus requiring a higher signal-to-noise ratio for its demonstration.

At variance with the results presented in Figures 2–5, nifurtimox and nitrofurazone radical anions gave multiline spectra (Figure 6) which recalled those obtained by Rao and Mason,[11] Moreno *et al.*[12] and Peterson *et al.*,[13] using as reductant catecholamine

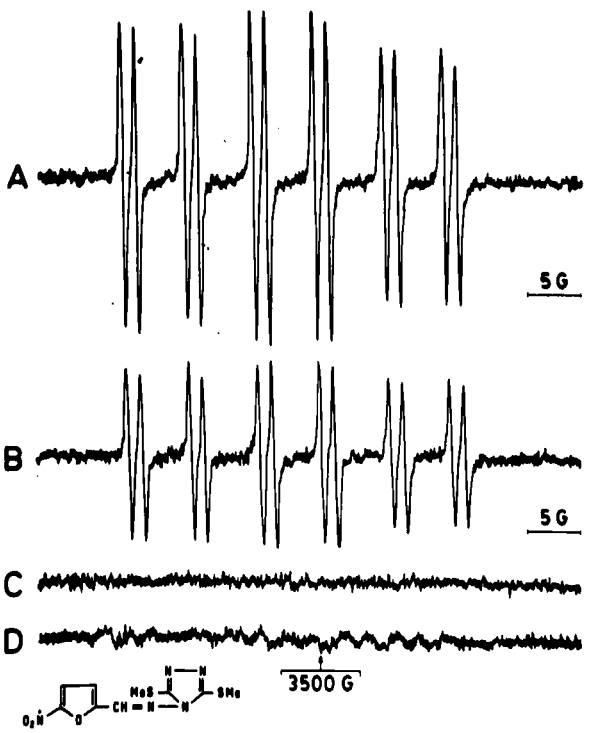

FIGURE 3 ESR spectra of radical anions produced in a system of NF-triazole(I) and 50 mM ascorbate in 0.1 N NaOH, under anaerobic conditions. Effect of nitrofuran concentration. A, complete system with 5.0 mM NF-triazole(I); B, same, with 2.5 mM NF-triazole(I); C, same without NF-triazole; D, same, with 2.5 mM NF-triazole, without ascorbate. Other experimental conditions were as described in Figure 2 legend.

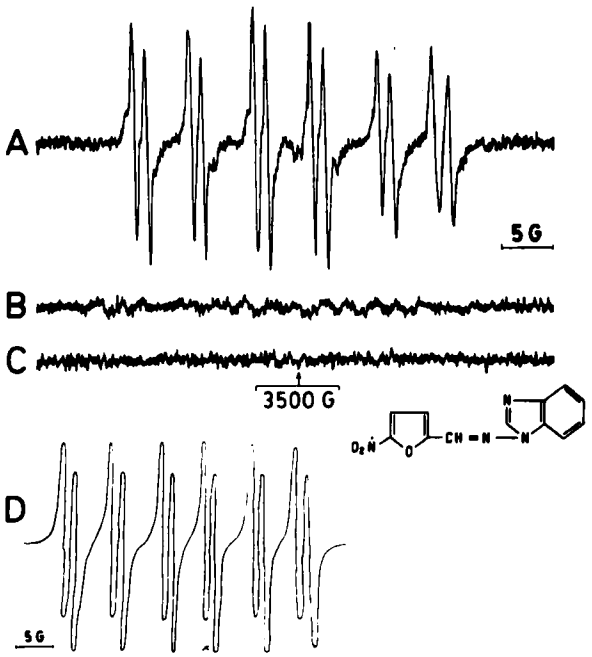

FIGURE 4 ESR spectra of radical anions produced in a system of 5.0 mM NF-benzimidazole and 50 mM ascorbate in 0.1 N NaOH, under anaerobic conditions, A, complete system; B, same, without ascorbate; C, same, without NF-benzimidazole; D, computer simulation of NF-benzimidazole anion radical spectrum (the HFSC values used were taken from Table I). Other conditions were as in Figure 2 legend.

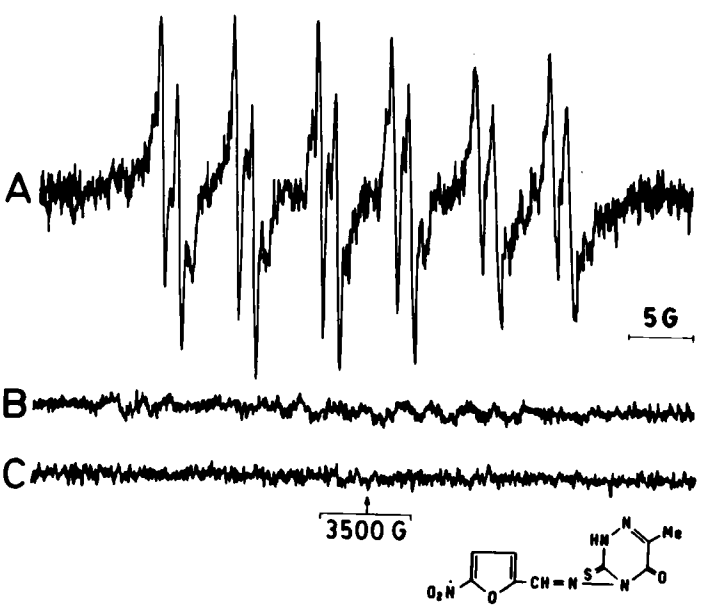

FIGURE 5 A, ESR spectum of NF-triazine radical anion. Gain, 2 × 10^6; other experimental conditions were as described in Figure 2 legend. B, sample without nitrofuran; C, sample without ascorbate.

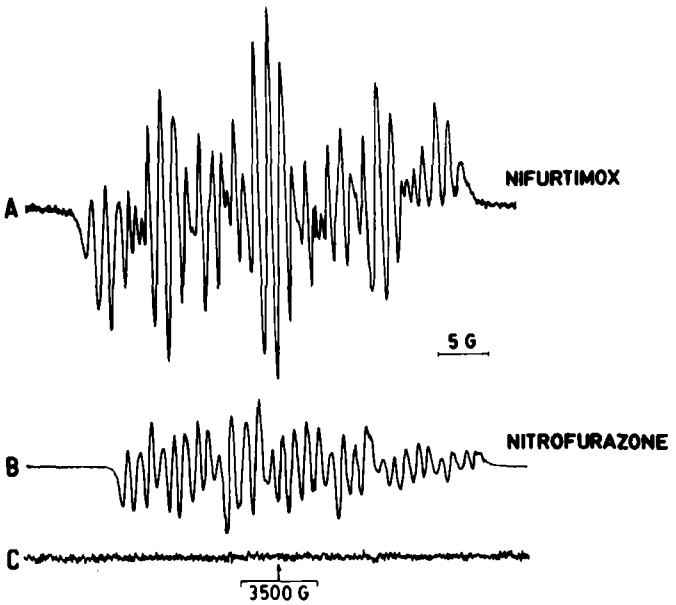

FIGURE 6 ESR spectrum of nifurtimox (A) and nitrofurazone (B) radical anions, Gain, 4×10^5 (A) or 8×10^4 (B). Other experimental conditions were as described in Figure 2 legend. C, sample without nitrofuran.

solutions at pH 9.5, substrate-supplemented mitochondria or bacterial nitroreductase at neutral pH, respectively.

Table I shows the HFSC assigned to nifurtimox analogues and related nitrofurans. It may be seen that with the former radicals, spin coupling was restricted to the nitrofuran group, but with nifurtimox, nitrofurazone and nitrofurantoin, coupling also occurred on the side chain of these compounds.

Oxygen consumption by solutions containing nitrofuran derivatives and ascorbate at neutral pH is a feature of radican anion production.[6] The nifurtimox analogues assayed were no exception to this rule, but their effect varied in accordance with the

TABLE I
Hyperfine spin coupling constants for nitro anion radicals

Nitro anion radical	Reducing system	HFSC constant (G)				
		1 N(NO_2)	1 H(4)	1 H(3)	1 H(CH = N)	1 N(CH = N)
NF-triazole	AA	12.40	5.80	1.35	ND	ND
NF-triazole (I)	AA	12.40	5.80	1.35	ND	ND
NF-benzimidazole	AA	12.40	5.80	1.35	ND	ND
NF-triazine	AA	12.40	5.80	1.35	ND	ND
Nitrofurantoin[6]	AA	11.03	5.91	1.53	1.00	2.30
Nifurtimox[11]	CA	11.00	6.05	1.61	1.16	2.35
Nitrofurazone[11]	CA	11.09	5.93	1.51	1.17	2.44

Experimental conditions were as described in Figs. 2 and 3 legends and in the Materials and Methods section. AA, ascorbate, at high pH; CA, catecholamine, at pH 9.5; ND, signal not detected.

TABLE II
Effect of nitrofuran compounds on ascorbate oxidation

Nitrofuran compound	Concentration (μM)	Nitrofuran molecular activity
NF-triazole	3–20	0.51 ± 0.04 (6)
NF-triazole(I)	2–20	0.46 ± 0.03 (3)
NF-triazole(II)	50	0.34 ± 0.02 (4)
NF-benzimidazole	50	0.25 ± 0.01 (3)
NF-pyrazole	10–20	0.24 ± 0.07 (3)
NF-triazine	10–50	0.19 ± 0.01 (4)
Nitrofurazone	200	0.033 ± 0.002(6)
Nifurtimox	300–1000	0.022 ± 0.001(5)
5-Nitro-2-furoic acid	200	0.014 ± 0.003(3)
None	– – –	0

The reaction mixture contained 5.0 mM sodium ascorbate, 1.0 mM DETAPAC, 0.1 mM Tris-HCl, pH 7.5, and nitrofuran. The latter was added dissolved in 2–20 μl DMFA. Each compound was assayed either at one or at several concentrations in the range indicated above. Other experimental conditions were as described in the Materials and Methods section. The results presented the average ± S.E.M. of the experimental values, which number is indicated by the figures in parentheses. Nitrofuran molecular activity: μmol O_2 uptake/min/μmol nitrofuran. DMFA did not stimulate oxygen uptake.

structure of the non-nitrofuran moiety (Table II). Thus, oxygen consumption was greatest with NF-triazole but smallest with nifurtimox and 5-nitro-2-furoic acid, a nearly forty-fold difference ($p < 0.05$) being observed between the most and the least active compounds. The decrease in the rate of oxygen consumption upon addition of superoxide dismutase and catalase (Table III) involved recovery of oxygen from the partially reduced intermediate, by catalase in the case of hydrogen peroxide, and by superoxide dismutase in the case of superoxide. Figure 7 shows the effect of increasing concentration of NF-triazole and nifurtimox on ascorbate oxidation. With nifurtimox, a linear correlation was obtained but with NF-triazole the molecular activity decreased at higher concentrations, in good agreement with results in Tables II and III.

Data in Tables I and II reflect the influence of the non-nitrofuran moiety on electron density distribution in the (5-nitro-2-furfurylidene) amino group. In this

TABLE III
Effect of superoxide dismutase and catalase on oxygen consumption by nifurtimox analogues in the presence of ascorbate

Nifurtimox analogue (100 μM)	Additions	Nitrofuran molecular activity	Decrease of oxygen consumption rate (%)
NF-triazole	None	0.33	– –
	SOD	0.17	50
	CAT	0.21	37
NF-benzimidazole	None	0.24	– –
	SOD	0.11	56
	CAT	0.15	38
None	None	0	– –

Unless stated otherwise, the experimental conditions were as described in Table II legend and in the Materials and Methods section. SOD, 500 U/ml; CAT, 5000 U/ml catalase. Nitrofuran molecular activity: μmol O_2 uptake/min/μmol nitrofuran.

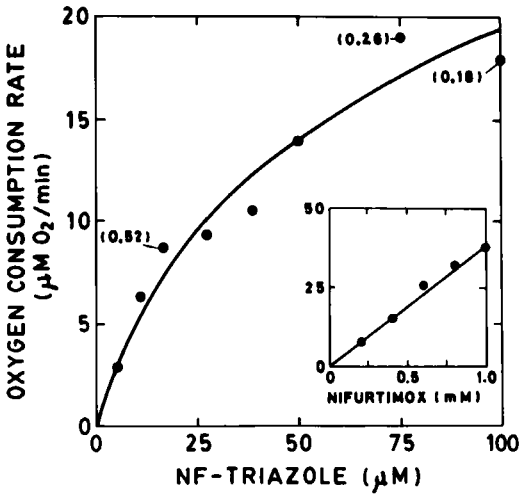

FIGURE 7 Effect of NF-triazole and nifurtimox concentrations on the rate of ascorbate oxidation. Experimental conditions were as described in Table II legend, except nitrofuran concentration which is indicated on the abscissa. The numbers in parentheses indicate NF-triazole molecular activity (μmol O_2 uptake/min/μmol nitrofuran).

context, the azomethine structure plays a central role, as indicated by NMR results in Table IV. It can be seen that with the triazole and triazine derivatives, the chemical shift value for the azomethine proton appeared at a lower field than with the thiazine and hydrazine carboxamide derivatives (nifurtimox and nitrofurazone, respectively), thus showing a strong deshielding action of the unsaturated nitrogen heterocycles.

DISCUSSION

Of the assigned HFSC given in Table I, the nitro group value may be used to classify the radical anions in two groups, namely, *a*) those having a 12.40 G constant, with an unsaturated nitrogen heterocycle moiety (NF-triazole, NF-triazole(I), NF-benzimidazole and NF-triazine) and *b*) those having a 11.00–11.09 G constant, with other moiety (nitrofurantoin, nifurtimox and nitrofurazone). As regards the former group

TABLE IV

Proton nuclear magnetic resonance of the (5-nitro-2-furfurylidene) amino group of nitrofuran compounds in DMFA-d_7

Nitrofuran compound	δ (ppm)		
	1 H(–CH = N–)	1 H(3)	1 H(4)
NF-triazole	9.27s	7.55d	7.88d
NF-triazine	9.07s	7.96d	8.09d
Nifurtimox	7.86s	6.95d	7.75d
Nitrofurazone	8.02s	7.79d	7.29d

Experimental conditions were as described in the Materials and Methods section. s, singlet; d, doublet. With all doublets, the coupling constant J was 3.5 Hz. Data for NF-triazole fit in well with those reported by Mester *et al.*[7] (9.12; 7.48; 7.72).

of nitrofuran compounds, it should be noted that all of them shared a common structural feature, namely, two nitrogen atoms in β-position (Figure 1). That structure would play an essential role for electron density distribution in the whole molecule.

Spin densities on nitro groups correlate with one-electron redox potentials (E_7^1) as illustrated by the equation $E_7^1 = 0.315 - 0.054a_{NO_2}^N$ where $a_{NO_2}^N$ is the HFSC constant for the nitro group.[14] This equation allowed an approximate calculation of E_7^1(s) for the compounds listed in Table I. The values obtained (in V) were -0.355 for nifurtimox analogues and -0.279, -0.284 and -0.281 for nifurtimox, nitro furazone and nitrofurantoin, respectively. The nitrofurazone potential approached -0.257 V, the value previously reported.[15]

The rate constant of the reaction of $R.NO_2^-$ with oxygen correlates with the one-electron potential of the nitro group[15] and the initial rate of that reaction is the faster, the more negative the value of $E_7^1(R.NO_2/R.NO_2^-)$. It seems then reasonable to assume that the more negative E_7^1 of nifurtimox analogues is the main cause of their greater activity on ascorbate oxidation. At neutral pH, secondary effects depending on the structure of the non-nitrofuran moiety may also affect nitrofuran capability for redox-cycling, as indicated by the different activities of NF-triazoles, NF-pyrazole, NF-benzimidazole and NF-triazine in Table II.

As regards nifurtimox analogues action on *T.cruzi*, a positive correlation can be established between oxyradical production (Table II) and growth inhibition percentages of culture epimastigote forms.[7] In fact, NF-pyrazole, NF-benzimidazole and the triazole derivatives were several times more potent than nifurtimox as inhibitors of parasite growth, in good agreement with their capability of generating superoxide anion (Table II). Accordingly, our observations support the notion that because of *T.cruzi* deficiency in enzymes preventing oxidative damage,[16–18] oxyradicals should be regarded as essential agents for the trypanocidal action of nitrofuran compounds.[4,19] Such correlation is not valid for their mutagenic action,[8] in which other mechanisms must be involved.

Acknowledgements

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References

1. R.P. Mason and J.L. Holtzman, (1975) The role of catalytic superoxide formation in the O_2 inhibition of nitroreductase. *Biochemistry Biophysics Research Communications*, **67**, 1267–1274.
2. R.P. Mason and J.L. Holtzman, (1975) The mechanism of microsomal and mitochondrial nitroreductase. Electron spin resonance evidence for nitroaromatic free radical intermemediates. *Biochemistry*, **14**, 1626–1632.
3. J.R. Ames, M.D. Ryan and P. Kovacic, (1986). Mechanism of antibacterial action: electron transfer and oxy radicals. *Journal of Free Radicals in Biological Medicine*, **2**, 377–391.
4. J.J. Marr and R. Docampo, (1986) Chemotherapy for Chagas' Disease: A perspective of current therapy and considerations for future research. *Reviews in Infection and Disease*, **8**, 884–903.
5. G.L. Kedderis and G.T. Miwa, (1988) The metabolic activation of nitroheterocyclic therapeutic agents. *Drug Metabolism Reviews*, **19**, 33–62.

6. D.N.R. Rao, L. Harman, A. Motten, J. Schreiber and R.P. Mason, (1987) Generation of radical anions of nitrofurantoin, misonidazole, and metronidazole by ascorbate. *Archives in Biochemistry and Biophysics,* **255,** 419–427.
7. B. Mester, J. Elguero, R.M. Claramunt, S. Castanys, M.L. Mascaró, A. Osuna, M.J. Vilaplana and P. Molina, (1987) Activity against *Trypanosoma cruzi* of new analogues of nifurtimox. *Archives in pharmacy (Weinhem),* **320,** 115–120.
8. E. Alejandre-Duran, R.M. Claramunt, D. Sanz, M.J. Vilaplana, P. Molina and C. Pueyo, (1988) Study on the mutagenicity of nifurtimox and eight derivatives with the L-arabinose resistance test of *Salmonella typhimurium. Mutation Research,* **206,** 193–200.
9. L. Grinblat, C.M. Sreider and A.O.M. Stoppani, (1989) Nitrofuran Inhibition of yeast and rat tissue glutathione reductases. Structure-activity relationships. *Biochemical Pharmacology,* **38,** 767–772.
10. H. Joela, (1988) A microcomputer program for second order simulation of EPR spectra. *Computers in Chemistry,* **12,** 189–206.
11. D.N.R. Rao and R.P. Mason, (1987) Generation of nitro radical anions of some 5-nitro-furans, 2- and 5-nitroimidazoles by norepinephrine, dopamine, and serotonin. A possible mechanism for neurotoxicity caused by nitroheterocyclic drugs. *Journal of Biological Chemistry,* **262,** 11731–11736.
12. S.J.N. Moreno, R.P. Mason and R. Docampo (1984) Reduction of nifurtimox and nitrofurantoin to free radical metabolites by rat liver mitochondria. *Journal of Biological Chemistry,* **259,** 6298–6305.
13. F.J. Peterson, R.P. Mason, J. Hovsepian and J.L. Holtzman (1979) Oxygen-sensitive and -insensitive nitroreduction by *Escherichia coli* and rat hepatic microsomes. *Journal of Biological Chemistry,* **254,** 4009–4014.
14. D. Meisel and P. Neta, (1975). One-electron redox potentials of nitro compounds and radiosensitizers. Correlation with spin densities of their radical anions. *Journal of the American Chemical Society,* **97,** 5198–5203.
15. P. Wardman and E.D. Clarke, (1976). Oxygen inhibition of nitroreductase: electron transfer from nitro radical-anions to oxygen. *Biochemistry and Biophysical Research Communications,* **69,** 942–949.
16. A. Boveris and A.O.M. Stoppani (1977) Hydrogen peroxide generation in *Trypanosoma cruzi. Experientia* **33,** 1306–1308.
17. R. Docampo, J.F. Boiso, A. Boveris and A.O.M. Stoppani, (1976). Localization of peroxide activity in *Trypanosoma cruzi* microbodies. *Experientia,* **32,** 972–975.
18. A. Boveris, H. Sies, E.E. Martino, R. Docampo, J.F. Turrens and A.O.M. Stoppani, (1980). Deficient metabolic utilization of hydrogen peroxide in *Trypanosoma cruzi. Biochemical Journal,* **188,** 643–648.
19. R. Docampo and A.O.M. Stoppani, (1979) Generation of superoxide anion and hydrogen peroxide induced by nifurtimox in *Trypanosoma cruzi. Archives in Biochemistry and Biophysics,* **197,** 317–321.

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