Bioorganic & Medicinal Chemistry 16 (2008) 7900–7907

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Furoxan-, alkylnitrate-derivatives and related compounds as anti-trypanosomatid agents: Mechanism of action studies

Lucía Boiani [a], Gabriela Aguirre [a], Mercedes González [a], Hugo Cerecetto [a,*], Agustina Chidichimo [b], Juan J. Cazzulo [b], Massimo Bertinaria [c,*], Stefano Guglielmo [c]

[a] Departamento de Química Orgánica, Facultad de Ciencias-Facultad de Química, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay
[b] Instituto de Investigaciones Biotecnológicas, Universidad Nacional de General San Martín-CONICET, 1650 San Martín, Buenos Aires, Argentina
[c] Dipartimento di Scienza e Tecnologia del Farmaco, Universitá degli Studi di Torino, Via P. Giuria, 10125 Torino, Italy

ARTICLE INFO

Article history:
Received 24 June 2008
Revised 24 July 2008
Accepted 25 July 2008
Available online 29 July 2008

Keywords:
Furoxan
Chagas disease
Nitric oxide

ABSTRACT

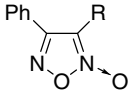
A series of over a hundred furoxans, alkylnitrates and related compounds were studied as growth inhibitors of the two major kinetoplastids of Latin America, *Trypanosoma cruzi*and *Leishmania* spp., in *in vitro* assays. The most active compounds showed 50% inhibitory doses of the same order of that of Nifurtimox and Miltefosine, reference drugs used to treat Chagas Disease and Leishmaniasis respectively. Among the studied compounds derivative **4**, presenting excellent inhibitory activity against the tryposmastigote and amastigote forms of *T. cruzi*, has emerged as a lead compound. Mechanism of action seems to involve mitochondrial dehydrogenases as a distinct effect with respect to Nifurtimox. Excreted metabolites, studied by NMR, showed a significant decrease in succinate, confirming the observed effect on the mitochrondrial dehydrogenases.

1. Introduction

Parasitic diseases affect hundreds of millions of people around the world, mainly in underdeveloped countries. Since parasitic protozoa are eukaryotic, they share many common features with their mammalian host making the development of effective and selective drugs a hard task. Diseases caused by *Trypanosomatidae*, which share a similar state regarding drug treatment, include Chagas' disease (*Trypanosoma cruzi*) and leishmaniasis (*Leishmania spp.*).[1] These trypanosomatids alone are responsible for an infected population of nearly 30 millions, and more than 400 millions are at risk. Drugs currently used in the treatment of Chagas' disease are two nitroaromatic heterocyles, Nifurtimox (Nfx, Lampit®, recently discontinued by Bayer) and Benznidazole (Bnz, Rochagan®, Roche), introduced empirically over three decades ago.[2] Both drugs are active in the acute phase of the disease, but efficacy is very low in the established chronic phase.

Moreover differences in drug susceptibility among different *T. cruzi* strains lead to varied parasitological cure rates according to the geographical area. The drugs of choice for the treatment of leishmaniasis are sodium stibogluconate (Pentostam®), meglumine antimoniate (Glucantime®), pentamidine and liposomal amphotericin B, but these sometimes meet with failure.[3] Currently, WHO/TDR develops a research program with Miltefosine (Mtf), a very promising leishmanocidal drug.[4] These illnesses, associated with poverty, do not attract the pharmaceutical companies as a result of the lack of commercial reasons; consequently, efforts to develop new and safer drugs have to be carried out mainly by academic institutions.

Endogenous nitric oxide (NO) is a potent antimicrobial agent. Together with reactive oxygen intermediates, NO is one of the toxic mediators released by activated macrophages against pathogens. NO-mediated cellular toxicity is due to the generation of reactive species and/or inhibition of essential enzymes.[5] In particular *S*-nitrosilation of cysteine containing proteins seems to be a widespread mechanism for the antiparasitic effect of NO.[6,7] A number of NO-donors, namely molecules able to release NO in physiological conditions like furoxans and organic nitrates, proved to display cytotoxic and cytostatic effects against viruses and microbial agents including protozoa such as *T. vaginalis*, *E. histolytica*, *T. cruzi*and *P. falciparum*.[8–12] In these last two cases we identified furoxans (1,2,5-oxadiazole 2-oxide derivatives), that is, **1** and **2** (Fig. 1),



-R= -CH=NNHCONH-hexyl, **1**
-SO_2Ph, **2**

Figure 1. Furoxan derivatives previously described as *T. cruzi* and *P. falciparum* growth inhibitors.

* Corresponding authors. Tel.: +598 2 5258618x216; fax: +598 2 5250749 (H.C.); tel.: +39 011 6707737; fax: +39 011 6707687 (M.B.).
E-mail addresses: hcerecet@fq.edu.uy (H. Cerecetto), massimo.bertinaria@unito.it (M. Bertinaria).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.07.077

with excellent growth inhibition capability probably as a result of their ability to release NO.

In the context of our research on the NO-donors pharmacochemistry, we decided to study selected derivatives as potential anti-*T. cruzi* and anti-*Leishmania* compounds. The 123 derivatives described in the present paper were selected from our furoxan-, furazan- and organic nitrate-chemical libraries in order to cover a wide structural spectrum. In this study NO-donor furoxans substituted with moieties endowed with different chemical and physico-chemical properties were used, together with their reduced analogues (furazans) devoid of the ability to release NO. In addition to antiparasitic activity we studied some biochemical changes promoted in the parasites by the most active compounds, that is, effects on the mitochondrial dehydrogenases, and on the excreted metabolites.

2. Methods and results

2.1. Selected compounds and synthesis

Two different series of NO-donors, furoxans (**2**–**17**, **31**–**50**, **61**–**62**, **64**–**68**, **72**–**78**, **85**–**86**, **88**–**96**, **104**–**110**, **112**–**113**) and alkylnitrates (**114**–**124**), were selected to analyze their anti-parasitic activity. In the first case, furazans (**18**–**30**, **51**–**60**, **63**, **69**–**71**, **79**–**84**, **87**, **97**–**103**, **111**) were also included as non-NO-donor analogues (Table 1). In some cases, antiparasitic-pharmacophores were included as substituents, as, for example, vinylsulfonyl[13] and 5-nitroimidazolyl[14] moieties (derivatives **3**, **19** and **32** and derivatives **15**, **27**, **40**, **53**, **66**, **70**, **74**, **80**, **89** and **98**, respectively, Table 1). The same structural features were covered in the derivatives belonging to alkylnitrate family (derivatives **114–124**, Table 2).

The studied compounds were prepared following synthetic procedures previously reported.[12,15] All the compounds were characterized by NMR (^{1}H and ^{13}C), IR and MS. The purity was established by TLC and microanalysis.

2.2. Biological characterization

*In vitroanti-T. cruzi*activity: The NO-donor derivatives were initially tested in vitro against the epimastigote form of *T. cruzi*, Tulahuen 2 strain. Recently, the existence of the epimastigote form of *T. cruzi*as an obligate mammalian intracellular stage has been revisited and confirmed.[16]The compounds were incorporated into the media at 25 μM, and their ability to inhibit growth of the parasite was evaluated in comparison to the control (no drug added to the media) at day 5. Nfx and Bnz were used as the reference trypanocidal drug. The percentage of growth inhibition (PGI) was calculated as indicated in Section 5. The ID_{50} concentrations (50% inhibitory concentration) were assessed for the most active derivatives, Nfx and Bnz (Tables 1 and 2). The structurally related furoxans and furazans **2–7**, **18–23**, **31–36**, **44**, **56** and **105** (Fig. 2) were further selected, among the most active derivatives, to be tested against the CL Brener clone and the Nfx- and Bnz-partially resistant Y strain.[17] In these last assays viability of *T. cruzi* was assessed colorimetrically using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay.[18] For each derivative, in a dose-response assay, between 1.0 and 50.0 μM, the ID_{50} concentration was calculated (Table 3).

Derivatives **3**, **4** and **6** were also evaluated, between 25.0 and 50.0 μM, against the bloodstream form of *T. cruzi* (trypomastigotes) of the CL Brener clone incubated during 2–24 h[19] (Table 4). The lysis percentage was evaluated against a control. Derivative **4**, devoid of toxicity against Vero cells at its ID_{50}against*T. cruzi* epimastigote form, was evaluated against the intracellular form of *T. cruzi* (amastigotes) of the CL Brener clone (Table 4).

In vitro leishmanocidal activity: The structurally related furoxans and furazans **2–7**, **18–23**, **31–36**, **44**, **56** and **105** (Fig. 2) were selected to be studied as leishmanocidal agents. They were tested in vitro against promastigote form of *L. braziliensis* (MHOM/BR/00/LTB300) and *L. pifanoi* (MHOM/VE/57/LV135) strains. Viability of parasite was assessed colorimetrically using the MTT assay.[20] For each derivative, the percentage of cytotoxicity was initially determined at 25 μM as it is indicated in Experimental Section and then in a dose-response assay, between 1.0 and 50.0 μM, the ID_{50} concentration was calculated and reported in Table 3. Mtf was used as the reference leishmanocidal drug.

2.3. Study of metabolic changes in the parasites

Derivatives **3**, **4** and **6** and Nfx were used to study the biochemical changes in the *T. cruzi*cell caused as a result of the action of these compounds. Two different aspects were studied: the effect of furoxans on mitochondrial dehydrogenases activities and modifications in the excreted metabolites.

For the first study, the percentage of mitochondrial dehydrogenase activities compared to untreated control was assessed using the colorimetric MTT assay performed at very short times, no more than 240 min of incubation, as described for *Leishmania* parasite.[21] Figure 3a shows the results for the studied compounds.

In order to study the changes in the biochemical pathways promoted by these furoxans, we studied the modifications in the excreted metabolites by ^{1}H NMR spectroscopy. This kind of studies proved to be a useful tool in the mechanism of action elucidation.[22] The study was performed comparing the ^{1}H NMR spectra of the cell-free medium of *T. cruzi* parasites treated with furoxans to those of the untreated parasites as the control.

In this study, we focused our attention mainly on the changes of the excreted salts of the carboxylic acids, lactate (Lac), acetate (Ace), pyruvate (Pyr) and succinate (Suc) and the aminoacids, alanine (Ala) and glycine (Gly), these being the most relevant metabolites in normal parasites (control, black columns, in Fig. 3b). It is well-known that *T. cruzi* sp. is incapable to degrade carbohydrates completely producing a mixture of CO_2, mono- and dicarboxylic acids, which are mainly Suc, Ala and Ace.[21,24] It was observed, by using enzymatic methods, that under aerobic conditions epimastigotes in culture produced mainly Lac, Pyr, Ace and Suc.[25] In our study, the three furoxans, **3**, **4** and **6**, induced a slight decrease of all the studied excreted metabolites with the exception of Ala and Pyr. A representative example of the changes in the excreted end-products after the treatment of epimastigote form of *T. cruzi*Y strain with derivative **3** is reported in Figure 3b (white columns).

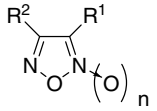
3. Discussion

In the present paper, we report the biological activity of nearly 120 compounds against epimastigote form of three different strains of *T. cruzi*, trypomastigote and amastigote forms of one strain of *T. cruzi* and two promastigote forms of two *Leishmania* species.

Furoxans **2–13**, **15–17**, **31–38**, **40**,**48**, **49**, **64**, **104**, and **108**, furazans **18–20**, **23**, **28** and **53**, and alkylnitrate **114** were the most potent derivatives against Tulahuen 2 strain being **2**, **11**, **15**, **16**, **28**, **34**, **40**, **53**, and **108** equi- or more potent than the reference drugs, Nfx and Bnz (Tables 1 and 2). The inclusion of 5-nitroimidazolyl-pharmacophore as substituent does not seem to be relevant for the anti-*T. cruzi* activity (compare activity of derivatives **64** and **66**, or see bio-response of derivatives **70**, **74**, **80**, **89**, **98**, **120**, and **121**).

In-depth biological studies were done with structurally related 3-sulfonyl derivatives, **2–7**, 4-sulfonyl derivatives, **31–36**, *N*-deoxygenated analogues (furazans), **18-23**, and thio-derivatives **44**, **56** and **105**, evaluating them against CL Brener clone, Y strain and *L. braziliensis*and*L. pifanoi*. In these studies, it was clearly established

Table 1
In vitro inhibition of epimastigote form of *T. cruzi* of furoxan and furazan derivatives

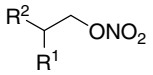


Compound	*n*	$-R^1$	$-R^2$	$-R^4$	$ID^{50a,b}$ ($PGI^{c,b}$)	Compound	*n*	$-R^1$	$-R^2$	$ID^{50a,b}$($PGI^{c,b}$)
1	1		[d]		~25.0[e]	**61**	1	$-R^{3f}$	–Me	(40.0)
2	1	$-SO_2R^4$	–Ph	–Ph	5.0	**62**	1	–CH=NOH	–Me	(17.0)
3	1	$-SO_2R^4$	–Ph	$-CH{=}CH_2$	6.5	**63**	0	$-R^{3f}$	–Me	(32.0)
4	1	$-SO_2R^4$	–Ph	$-CH_2CH_2OEt$	10.0	**64**	1	–CN	–Ph	10.0
5	1	$-SO_2R^4$	–Ph	$-CH_2CH_2SEt$	7.5	**65**[g]	1	–CN	$-CH_2N(Me)_2$	(5.0)
6	1	$-SO_2R^4$	–Ph	$-CH_2CONH_2$	10.0	**66**	1	–CN	$-CH_2NHCH_2CH_2R^{5h}$	(5.0)
7[g]	1	$-SO_2R^4$	–Ph	$-CH_2CH_2NH$(Ph-*o*-OMe)	7.5	**67**[i]	1	–CN	$-CH_2NHCHR^6R^{7j}$	(7.0)
8	1	$-SO_2R^4$	–Ph	$-CH_2CON(Me)_2$	7.5	**68**[k]	1	–CN	$-CH_2R^{8l}$	(9.0)
9	1	$-SO_2R^4$	–Ph	$-CH_2CH_2OH$	7.5	**69**[g]	0	–CN	$-CH_2N(Me)_2$	(13.0)
10	1	$-SO_2R^4$	–OEt	–Ph	7.5	**70**	0	–CN	$-CH_2NHCH_2CH_2R^{5h}$	(23.0)
11[g]	1	$-SO_2R^4$	$-O(CH_2)_3N(Me)_2$	–Ph	3.0	**71** [m]	0	–CN	$-CH_2R^{8l}$	(0.0)
12	1	$-SO_2R^4$	$-O(CH_2)_3OH$	–Ph	7.5	**72**	1	$-CONH_2$	–Ph	(35.0)
13	1	$-SO_2R^4$	$-OCH_2CO_2Et$	–Ph	7.5	**73**[g]	1	$-CONH_2$	$-CH_2N(Me)_2$	(11.0)
14	1	$-SO_2R^4$	$-OCH_2CO_2H$	–Ph	(35.0)	**74**	1	$-CONH_2$	$-CH_2NHCH_2CH_2R^{5h}$	(10.0)
15	1	$-SO_2R^4$	$-OCH_2CH_2R^{5h}$	–Ph	5.0	**75**[n]	1	$-CONH_2$	$-CH_2$(1-piperidinyl)	(4.0)
16	1	$-SO_2R^4$	$-SO_2R^4$	–Ph	3.5	**76**[i]	1	$-CONH_2$	$-CH_2NHCHR^6R^{7j}$	(17.0)
17	1	$-SO_2R^4$	–Me	–Ph-*p*-F	7.5	**77**[o]	1	$-CONH_2$	$-CH_2R^{8l}$	(6.0)
18	0	$-SO_2R^4$	–Ph	–Ph	10.0	**78**	1	$-CONH_2$	$-CH_2OH$	(0.0)
19	0	$-SO_2R^4$	–Ph	$-CH{=}CH_2$	6.5	**79**[g]	0	$-CONH_2$	$-CH_2N(Me)_2$	(0.0)
20	0	$-SO_2R^4$	–Ph	$-CH_2CH_2OEt$	7.5	**80**	0	$-CONH_2$	$-CH_2NHCH_2CH_2R^{5h}$	(14.0)
21	0	$-SO_2R^4$	–Ph	$-CH_2CH_2SEt$	(25.0)	**81**[n]	0	$-CONH_2$	$-CH_2$(1-piperidinyl)	(3.0)
22	0	$-SO_2R^4$	–Ph	$-CH_2CONH_2$	(27.0)	**82**[p]	0	$-CONH_2$	$-CH_2NHCHR^6R^{7j}$	(0.0)
23	0	$-SO_2R^4$	–Ph	$-CH_2CH_2NH$(Ph-*o*-OMe)	10.0	**83**[q]	0	$-CONH_2$	$-CH_2R^{8l}$	(0.0)
24	0	$-SO_2R^4$	–Ph	$-CH_2CH_2OH$	(17.0)	**84**	0	$-CONH_2$	$-CH_2OH$	(0.0)
25	0	$-SO_2R^4$	–OEt	–Ph	(25.0)	**85**	1	–CO(piperidinyl)	$-NH_2$	(17.0)
26[g]	0	$-SO_2R^4$	$-O(CH_2)_3N(Me)_2$	–Ph	(15.0)	**86**	1	$-CONHR^{9r}$	–Me	(10.0)
27	0	$-SO_2R^4$	$-OCH_2CH_2R^{5h}$	–Ph	19.0	**87**	0	$-CONHR^{9r}$	–Me	(16.0)
28	0	$-SO_2R^4$	$-SO_2R^4$	–Ph	2.0	**88**[g]	1	–Me	$-CH_2N(Me)_2$	(0.0)
29	0	$-SO_2R^4$	–Me	–Ph-*p*-F	(21.0)	**89**	1	–Me	$-CH_2NHCH_2CH_2R^{5h}$	(17.0)
30[g]	0	$-SO_2R^4$	–4-imidazolyl	–Ph	(12.0)	**90**[s]	1	–Me	$-CH_2NHCHR^6R^{7j}$	(0.0)
31	1	–Ph	$-SO_2R^4$	–Ph	7.5	**91**[t]	1	–Me	$-CH_2R^{8l}$	(4.0)
32	1	–Ph	$-SO_2R^4$	$-CH{=}CH_2$	5.5	**92**	1	–Me	$-R^{3f}$	(29.0)
33	1	–Ph	$-SO_2R^4$	$-CH_2CH_2OEt$	17.0	**93**	1	–Me	–CH=NOH	(32.0)
34	1	–Ph	$-SO_2R^4$	$-CH_2CH_2SEt$	5.0	**94**	1	–Me	$-CONHR^{9r}$	(9.0)
35	1	–Ph	$-SO_2R^4$	$-CH_2CONH_2$	25.0	**95**	1	–Me	–Ph-*p*-CO_2H	(34.0)
36	1	–Ph	$-SO_2R^4$	$-CH_2CH_2NH$(Ph-*o*-OMe)	8.0	**96**	1	–Me	$-SO_2$Ph-*p*-F	(44.0)
37	1	–Ph	$-SO_2R^4$	$-CH_2CH_2OH$	25.0	**97**[g]	0	–Me	$-CH_2N(Me)_2$	(0.0)

38[g]	1	–Ph	–OEt	–	7.5	**98**	0	–Me	$-CH_2NHCH_2CH_2R^{5h}$	(18.0)
39	1	–Ph	$-O(CH_2)_3N(Me)_2$	–	(0.0)	**99**[s]	0	–Me	$-CH_2NHCHR^6R^{7j}$	(2.0)
40	1	–Ph	$-OCH_2CH_2R^{5h}$	–	4.0	**100**[u]	0	–Me	$-CH_2R^{8l}$	(21.0)
41	1	–Ph	$-O(CH_2)_2SO_2Me$	–	(11.0)	**101**	0	–Me	benzothiazol-2-yl	(22.0)
42	1	–Ph	$-SCH_2CO_2Me$	–	(15.0)	**102**	0	–Me	benzoimidazol-2-yl	(20.0)
43	1	–Ph	$-SCH_2CO_2H$	–	(4.0)	**103**	0	–Me	benzoxazol-2-yl	(15.0)
44	1	–Ph	$-SCH_2CONH_2$	–	(12.0)	**104**	1	–OEt	–Ph	18.0
45[n]	1	–Ph	–S-(4-piperidinyl)	–	(32.0)	**105**	1	$-SCH_2CONH_2$	–Ph	(19.0)
46	1	–Ph	$-CONH_2$	–	(10.0)	**106**[g]	1	–S-(4-piperidinyl)	–Ph	(35.0)
47	1	–Ph	–CN	–	(15.0)	**107**	1	$-NH_2$	–Ph	(39.0)
48	1	–Ph	$-NH_2$	–	25.0	**108**	1	–Br	–Ph	5.0
49	1	–Ph	–Br	–	13.0	**109**	1	$-SCH_2CO_2Me$	$-SO_2Ph$	(22.0)
50	1	–Ph-*p*-CO_2H	–Me	–	(38.0)	**110**	1	$-SCH_2CO_2H$	$-SO_2Ph$	(0.0)
51	0	–Ph	–OEt	–	(35.0)	**111**[v]	0	$-O(CH_2)_3N(Me)_2$	–4-imidazolyl	(3.0)
52[g]	0	–Ph	$-O(CH_2)_3N(Me)_2$	–	(0.0)	**112**	1	–CH=NOH	$-CH_2OH$	(2.0)
53	0	–Ph	$-OCH_2CH_2R^{5h}$	–	5.0	**113**	1	$-CH_2OH$	–CH=NOH	(3.0)
54	0	–Ph	$-O(CH_2)_2SO_2Me$	–	(4.0)					
55	0	–Ph	$-SCH_2CO_2H$	–	(31.0)	**Nfx**	–	–	–	7.7
56	0	–Ph	$-SCH_2CONH_2$	–	(17.0)	**Bnz**	–	–	–	4.5
57[g]	0	–Ph	–S-(4-piperidinyl)	–	(34.0)					
58	0	–Ph	–CN	–	(2.0)					
59	0	–Ph	$-NH_2$	–	(45.0)					
60	0	–Ph-*p*-CO_2H	–Me	–	(32.0)					

[a] ID_{50} in μM.
[b] The results are the means of three independent experiments with a SD less than 10% in all cases.
[c] Percentage of growth inhibition of epimastigotes of Tulahuen 2 strain at 25 μM.
[d] See structure in Figure 1.
[e] From Ref. 11
[f] $-R^3$: –(3-mercapto-1,2,4-triazol)-5-yl.
[g] As HCl.
[h] $-R^5$: –2-methyl-5-nitroimidazol-1-yl.
[i] As $2CF_3CO_2H$.
[j] $-R^6$: –4-imidazolyl; $-R^7$: –4-piperidinyl.
[k] As $1.5C_2H_2O_4$.
[l] $-R^8$: –4-(4-imidazolylmethyl)piperidinyl.
[m] As $1.5C_2H_2O_4{\cdot}0.2H_2O$.
[n] As $C_2H_2O_4$.
[o] As $1.5C_2H_2O_4H_2O$.
[p] As $2CF_3CO_2H{\cdot}H_2O{\cdot}0.1Et_2O$.
[q] As $C_2H_2O_4{\cdot}0.5\ H_2O$.
[r] $-R^9$: $-NHC(S)NH_2$.
[s] As $2CF_3CO_2H{\cdot}0.5H_2O$.
[t] As $0.5C_2H_2O_40.2\ H_2O$.
[u] As $2C_2H_2O_4$.
[v] As 2HCl.

Table 2
Biological characterization of alkylnitrate derivatives



Compound	$-R^1$	$-R^2$	ID_{50}[a,b]($PGI^{c,b}$)	Compound	$-R^1$	$-R^2$	$ID^{50a,b}$($PGI^{c,b}$)
114[d]	$-ONO_2$	$-CH_2NH_2$	25.0	**120**[e]	$-ONO_2$	$-CH_2OCH_2CH_2$-(2-methyl-5-nitroimidazol-1-yl)	(10.0)
115[f]	$-ONO_2$	$-CH_2Ph$-*p*-R[4g]	(3.0)	**121**[e]	–H	$-OCH_2CH_2$-(2-methyl-5-nitroimidazol-1-yl)	(13.0)
116	$-ONO_2$	$-CH_2OPh$-*p*-CHO	(1.0)	**122**[d]	–H	$-CH_2NH_2$	(0.0)
117	$-ONO_2$	$-CH_2OPh$-*p*-CO_2H	(0.0)	**123**[d]	–H	$-CH_2NHEt$	(0.0)
118	$-ONO_2$	$-CH_2Ph$-*p*-CHO	(0.0)	**124**[d]	–Me	$-CH_2NHCH_2CH(CH_3)ONO_2$	(0.0)
119	$-ONO_2$	$-CH_2Ph$-*p*-CO_2H	(0.0)				

[a] ID_{50} in μM.
[b] The results are the means of three independent experiments with a SD less than 10% in all cases.
[c] Percentage of growth inhibition of epimastigotes of Tulahuen 2 strain at 25 μM.
[d] As HNO_3.
[e] As HCl.
[f] As 2HCl.
[g] $-R^4$: –4-[(3,4,5-trimethoxyphenyl)methyl]piperazin-1-ylmethyl.

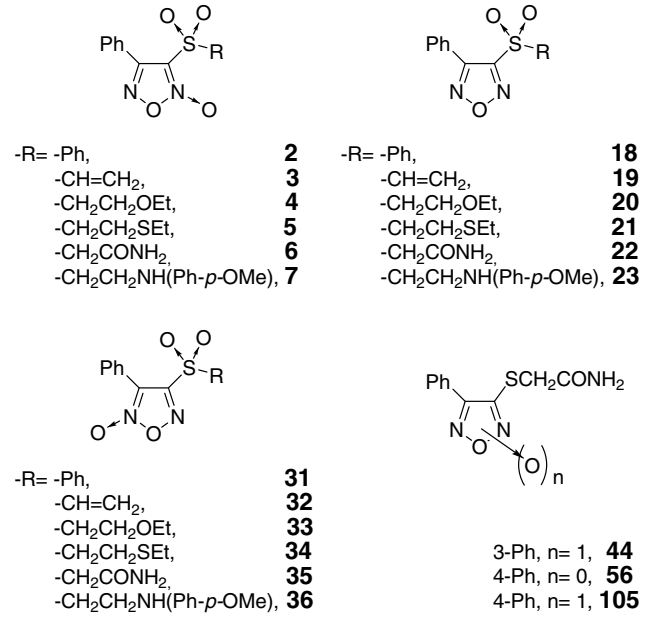


Figure 2. Furoxan and furazan derivatives selected for in-depth biological studies.

that derivatives **2-7** are the most active compounds (Table 3). Additionally, activities of compounds **3**, **4**, and **6** against the trypomastigote and amastigote forms of the CL Brener clone were maintained (Table 4). Furoxan **4** has an endocytic index, corresponding to the percentage of infected Vero cells multiplied by the average number of intracellular amastigotes (EI, Table 4), nearly sevenfold lower than that of the untreated control. This compound could be considered an excellent lead for further biological studies. From a structural point of view, in general the most active family is that bearing a 3-sulfonyl substituent, while the 4-sulfonyl isomers have medium activities. The absence of the *N*-oxide moiety or the substitution by an alkylthio group induces loss of activity. The high activity of 3-sulfonyl furoxans **2-7** with respect to that of the corresponding 4-sulfonyl isomers **31**–**36** parallels their capacity of releasing NO under the action of thiols (Table 5), and this suggest a possible role of this species in their toxicity against the studied parasites. This hypothesis seems to be supported by the partial loss of activity of the furazan analogues **18**–**23** devoid of this capacity. Another possibility is that the activity is (or is also) dependent on the block of thiol groups essential for the parasite life. Additional studies are necessary to clarify this point.

Table 3
ID_{50} values of selected Furoxans and Furazans against different *T. cruzi* and *Leishmania* spp. strains

Compound	ID_{50}[a] (μM)			
	T. cruzi CL Brener clone	*T. cruzi* Y strain	*L. braziliensis* LTB300	*L. pifanoi* LV135
2	4.2	4.3	1.6	3.8
3	2.6	6.8	10.0	26.7
4	1.5	3.4	4.1	4.2
5	0.9	3.9	4.0	4.6
6	3.7	6.0	14.3	>50.0
7	5.0	9.2	13.7	6.5
18	>50.0	>50.0	>50.0	>50.0
19	22.2	23.5	8.0	20.4
20	>50.0	>50.0	>50.0	>50.0
21	>50.0	28.7	>50.0	>50.0
22	>50.0	>50.0	>50.0	>50.0
23	>50.0	50.0	11.6	15.8
31	9.1	>50.0	20.2	20.5
32	8.2	11.1	39.1	>50.0
33	>50.0	>50.0	>50.0	>50.0
34	37.1	6.0	>50.0	>50.0
35	>50.0	48.0	>50.0	>50.0
36	7.2	26.2	9.9	33.8
44	>50.0	>50.0	>50.0	>50.0
56	>50.0	>50.0	>50.0	>50.0
105	>50.0	>50.0	>50.0	>50.0
Nfx	4.9	9.7	–	–
Mtf	–	–	9.0	(85.5[b])

[a] The results are the means of three independent experiments with a SD less than 10% in all cases.
[b] Compound tested at 5.0 μM, % of growth inhibition is reported.

In the metabolic changes studies, some relevant features could be observed. On the one hand, unlike Nfx, the studied furoxans **3**, **4** and **6**, affect mitochondrial dehydrogenases, their activities being lower than that of the untreated (taken as 100%) or Nfx-treated parasites (Fig. 3a). On the other hand, generally speaking, furoxans **3**, **4** and **6** promoted a slight reduction of the studied end-metabolites concentrations with respect to untreated parasites (Fig. 3b). The most relevant reduction was observed for Suc, and this could reflect the furoxans capability to act as mitochondrial dehydrogenases inhibitors. Since mitochondrial Suc-dehydrogenase (SDH) and fumarate reductases (FRDs) are enzymes endowed with a high degree of homology[26] it is likely that most SDH inhibitors could also act by inhibiting FRDs, and this could be the case with our furoxan derivatives. In trypanosomatids FRDs are located in at least two different compartments, namely mitochondrion and glycosome,[26b] and dihydroorotate dehydrogenase (DHOD) could also act as a sol-

Table 4
In vitro trypanocidal activity against trypomastigote and amastigote forms of furoxans **3**, **4** and **6**

Compound	Doses (μM)	Time (h)	% Lysis[a,b]
Trypomastigotes			
3	25.0	2	33
		19	100
	50.0	2	62
		19	100
4	25.0	19	100
	50.0	2	68
		19	100
6	10.0	2	0
		3	5
		26	100
	25.0	19	100
	50.0	19	100
	PIC (%)[c]	NAC[d]	EI[e]
Amastigotes			
4[c]	6.3	1.6	10.1
C[f]	23.3	3.2	74.6

[a] % Lysis, percentage of parasite lysis at the assayed doses.
[b] Results are the mean of three independent experiments with a SD less than 10% in all cases.
[c] PIC, percentage of amastigote-infected Vero cells (for details, see Section 5).
[d] NAC, number of amastigotes per infected Vero cells (for details, Section 5).
[e] EI, endocytic index (for details, see Section 5). The compound was tested at 2.5 μM.
[f] C, control; untreated Vero cells infected with amastigotes.

uble FRD.[26c] The inhibition of the mitochondrial FRD promotes the depletion of fumarate with concomitant decrease in Suc production. On the other hand inhibition of glycosomal FRD or DHOD should also lead to a decrease in Suc excretion, consequently an action of furoxans at these levels cannot be excluded. Further studies should be planned to clarify this issue.

4. Conclusions

In conclusion, we have identified furoxans as promising anti-trypanosomatid agents. In particular, together with lead compound **4**, other interesting derivatives namely, **2**, **3**, **5** and **6** have emerged from this work, and they could be object of further structural modifications and further biological studies on both *Trypanosome* and *Leishmania* parasites.

Table 5
In vitro anti-parasitic activity of studied furoxans and furazans and thiol-dependent nitrite production

Compound	$\%NO_2^-$ ± SE[a]	Anti-parasitic activity[b]
2	10.3 ± 0.1	+++
4	10.0 ± 0.5	+++
5	10.0 ± 0.4	+++
7	20.8 ± 0.7	+++
31	<1	+
33	<1	–
34	<1	+
36	<1	++
18	ND[c]	–
20	ND	–
21	ND	–
23	ND	+

[a] $\%NO_2^-$: percentage of nitrite production (mol/mol) determined by Griess assay.[12]
[b] +++, high activity against *T. cruzi* and *Leishmania*; ++, medium activity against both parasites; +, medium activity against one parasite; –, inactive against both parasites.
[c] ND, not detected.

5. Experimental

5.1. Chemistry

All starting materials were commercially available research-grade chemicals, and were used without further purification. Compounds **3–5**, **7**, **9**, **19-21**, **23–24**, **32–34**, **36–37**,[12] **2**, **18**, **31**,[15a] **6**, **22**, **35**, **41–44**, **54–56**, **105**,[15b] **38**, **10**, **104**,[15c] **15**, **25**, **27**, **40**, **51**, **53**, **66**, **70**, **74**, **80**, **89**, **98**, **120-121**,[15d],**16**[15e] **28**,[15f] **11**, **64**,[15g] **39**,[15h] **12**,[15i] **17**, **29**, **96**,[15j] **45**, **57**, **106**,[15k] **46–47**, **72**,[15l] **48**, **59**, **107**,[15m] **49**, **108**,[15n] **50**, **60**, **95**,[15o] **58**,[15p] **68**,[15q] **78**,[15r] **84**,[15s] **85**,[15t] **86-87**, **94**,[15u] **62**, **93**, **112–113**,[15v] **13–14**, **109-110**, **116–119**,[15w] **122**,[15x] **114**,[15y] **8**, **26**, **30**, **52**, **61**, **63**, **67–69**, **71**, **73**, **75–77**, **79**, **81–83**, **88**, **90–92**, **97**, **99–100**, **101–103**, **111**, **121**, **123–124**[15z] were prepared following synthetic procedures previously reported.

5.2. Biology

5.2.1. Anti-trypanosomatid in vitro evaluation

5.2.1.1. Anti-*T. cruzi* in vitro test using epimastigotes of Tulahuen 2 strain. *Trypanosoma cruzi* epimastigotes (Tulahuen 2 strain) were grown at 28 °C in an axenic medium (BHI-Tryptose) as previously described,[19,27] supplemented with 5% foetal bovine serum (FBS). Cells from a 10-day-old culture (stationary phase)

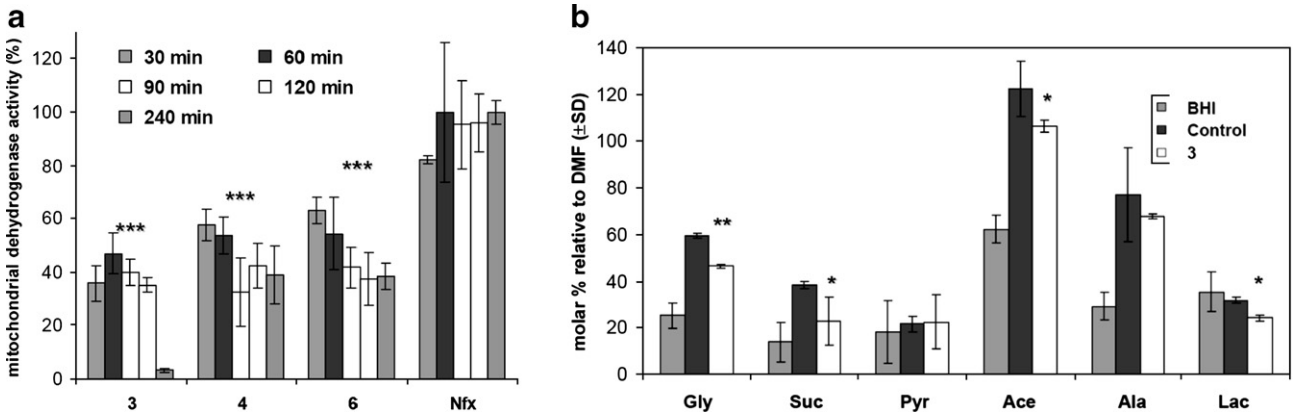


Figure 3. (a) Mitochondrial dehydrogenases activities (%) compared to untreated control of *T. cruzi* epimastigote, Y strain, with time and compound (for details, see Section 5). (b) Percentage of the end-products excreted in the medium after treatment of *T. cruzi* epimastigote Y strain with derivative **3** (white column) compared to untreated epimastigotes (black column). Culture medium, BHI-Tryptose, without parasite is included as reference of the end-products basal levels (grey column). Results are expressed as molar % relative to DMF, used as internal standard (for details, see Section 5).[23] Results are presented as means ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the respective control.

were inoculated into 50 mL of fresh culture medium to give an initial concentration of 1×10^6 cells/mL. Cell growth was followed by measuring everyday the absorbance of the culture at 600 nm. Before inoculation, the media were supplemented with the indicated amount of the drug from a stock solution in DMSO. The final concentration of DMSO in the culture media never exceeded 0.4%, and the control was run in the presence of 0.4% DMSO and in the absence of any drug. No effect on epimastigote growth was observed by the presence of up to 1% DMSO in the culture media. The percentage of inhibition (PGI) was calculated as follows: PGI (%) = $\{1 - [(Ap - A0p)/(Ac - A0c)]\} \times 100$, where Ap = A_{600} of the culture containing the drug at day 5; A0p = A_{600} of the culture containing the drug just after addition of the inocula (day 0); Ac = A_{600} of the culture in the absence of any drug (control) at day 5; A0c = A_{600} in the absence of the drug at day 0. To determine ID_{50} values, 50% inhibitory concentrations, parasite growth was followed in the absence (control) and presence of increasing concentrations of the corresponding drug. At day 5, the absorbance of the culture was measured and related to the control. The ID_{50} value was taken as the concentration of drug needed to reduce the absorbance ratio to 50%.

5.2.1.2. Anti-*T. cruzi* in vitro test using trypomastigotes and amastigotes of the CL Brener clone[28]. Vero cell cultures infected with cell culture trypomastigotes were incubated at 37 °C in humidified air with 5% CO_2 for 5–7 days. After this time, the culture medium was collected and centrifuged at 4000***g*** for 10 min, and the trypomastigote-containing pellet was left at 37 °C for 4 h, to allow the trypomastigotes to swim off. The remaining pellet was discarded, and this supernatant was used for the experiments. After trypomastigotes exposure to different doses and in different periods of the studied furoxans the percentage of lysis was determined in reference to untreated parasites. The percentage of lysis was determined using Neubauer counting chamber.

Vero cells (4×10^7/L) were cultured at 37 °C in modified Eagle's medium containing 3% (v/v) FBS, in 24-well plates containing glass coverslips. After 24 h the cultures were inoculated with CL Brener clone cell culture trypomastigotes (5×10^9/L) with or without compound **4**. After 24 h, the medium containing the non-internalized parasites was removed; fresh medium, with or without compound **4**, was added, and the infected cells were incubated for 72 h and stained with May–Grunwald–Giemsa. The percentage of infected cells and the number of intracellular parasites were estimated by observing 500 cells in a Nikon Eclipse E400 microscope. The results are expressed as the endocytic index (product of the percent of infected cells and the number of amastigotes per cell).

5.2.1.3. Viability of the CL Brener clone or Y strain of *T. cruzi* and LTB300 strain of *L. braziliensis* and LV135 strain of *L. pifanoi*

Trypanosoma cruzi epimastigotes (CL Brener or Y strain) were grown as is indicated in Section 5.2.1.1. *Leishmania braziliensis*(MHOM/BR/00/LTB300 strain) and *Leishmania pifanoi* (MHOM/VE/57/LV135) promastigotes were grown at 28 °C in an axenic-RPMI medium supplemented with 5% FBS as previously described.[19] Cell-culture plates consisting of 24 wells were filled at 1 mL/well with the corresponding parasite strain culture during its exponential growth in the corresponding medium. BHI-Tryptose medium was supplemented with 5% foetal bovine serum (FBS). Different doses of studied compounds dissolved in DMSO were added and maintained for 5 days. Afterwards, the cells were washed with PBS and incubated (37 °C) with 0.4 mg/mL MTT (Sigma) for 3 h. Then, formazan was dissolved with DMSO (180 μL), and optical densities were measured. Each concentration was assayed three times, and six growth controls were used in each test. Cytotoxicity percentages (PCyt (%)) were determined as follows: PCyt = $[100 - (ODd - ODdm)/(ODc - ODcm)] \times 100$, where ODd is the mean of OD595 of wells with parasites and different concentrations of the compounds, ODdm is the mean of OD595 of wells with different compound concentrations in the medium, ODc is the growth control, and ODcm is the mean of OD595 of wells with medium only. The ID_{50} value was taken as the concentration of drug needed to reduce the absorbance ratio to 50%.

5.2.2. Study of metabolic changes in the parasites

5.2.2.1. Mitochondrial dehydrogenase activities. Mitochondrial dehydrogenase activities were measured in 24-well plates. One million of ***T. cruzi*** epimastigotes (Y strain) in 500 μL medium were seeded in each well, and 20 μM of studied compounds was added. Two wells with untreated parasites were maintained as controls corresponding to the given time of treatment. The cultures were incubated at 28 °C. At the different time incubations, the epimastigotes were counted, and the colorimetric MTT dye-reduction assay was performed, the tetrazolium salt being converted into purple formazan by mitochondria. Fifty microlitres of a solution containing 5 mg/mL of MTT in PBS were added to each well, and plates were incubated for an additional 4 h. The reaction was stopped by addition of 500 μL of acidic isopropanol (0.4 mL HCl of 10 ***N*** in 100 mL isopropanol). The absorbance was measured at 570 nm. Under our conditions, compounds did not interfere with the reaction mixture. Percentage of mitochondrial dehydrogenase activities (%) was determined using untreated parasites-activities as 100%. Results are expressed as the mean.± standard error of the mean (SEM), and comparisons were made using Student's ***t*** test. A probability of 0.05 or less was considered significant.

5.2.2.2. ^{1}H NMR study of the excreted metabolites

For the spectroscopic studies, 5 mL of a 2-day-treated *T. cruzi* (Y strain) with each studied compound (5 μM) was centrifuged at 1500*g* for 10 min at 4 °C. The pellet was discarded, and the parasite-free supernatant was stored at −20 °C until used. Before the measurement, about 0.1 mL of DMF (10 mM) as internal standard, and 0.1 mL of D_2O were added to 0.3 mL of the supernatant. The spectra were registered with water suppression in 5 mm NMR sample tubes. The chemical displacements used to identify the respective metabolites were confirmed by both adding each analyzed metabolite to the studied supernatant and the study of a control solution with 4 μg/mL of each metabolites in buffer phosphate, pH 7.4. Results are expressed as molar percentage relative to DMF, used as internal standard. Results are expressed as the mean.± standard error of the mean (SEM), and comparisons were made using Student's *t* test. A probability of 0.05 or less was considered significant.

Acknowledgments

The authors thank the CSIC-Universidad de la República. L.B. thanks PEDECIBA (Uruguay) for her scholarship. We thank IUBMB for a fellowship to L.B. J.J.C. is a member of the Research Career of the Argentinean National Research Council (CONICET), and A.C. has a fellowship for the Universidad Nacional de General San Martin. Authors also thank Professor Alberto Gasco for supplying some compounds and for helpful discussion.

References and notes

1. World Health Organization, Thirteenth Program Report, UNDP/World Bank/World Health Organization program for research and training in tropical diseases, World Health Organization: Geneva, **1997**.
2. Cerecetto, H.; González, M. *Curr. Top. Med. Chem.* **2002**, *2*, 1187.
3. Berman, J. *Curr. Opin. Infect. Dis.* **2003**, *16*, 397.

4. (a) Guerin, P. J.; Olliaro, P.; Sundar, S.; Boelaert, M.; Croft, S. L.; Desjeux, P.; Wasunna, K.; Bryceson, A. D. M. *Lancet Infect. Dis.* **2002**, *2*, 494; (b) Rosenthal, E.; Marty, P. *J. Postgrad. Med.* **2003**, *49*, 61.
5. Fang, F. C. *J. Clin. Invest.* **1997**, *99*, 2818.
6. Colasanti, M.; Gradoni, L.; Mattu, M.; Persichini, T.; Salvati, L.; Venturini, G.; Ascenzi, P. *Int. J. Mol. Med.* **2002**, *9*, 131.
7. Ascenzi, P.; Bocedi, A.; Gentile, M.; Visca, P.; Gradoni, L. *Biochim. Bhiophys. Acta* **2004**, *1703*, 69.
8. Benz, D.; Cadet, P.; Mantione, K.; Zhu, W.; Stefano, G. B. In *Biochemical Significance of Nitric Oxide*; Stefano, G. B., Ed.; MSI: Warsaw/NewYork, 2003; pp 389–394. and references therein.
9. Rockett, K. K.; Awburn, M. M.; Cowden, W. B.; Clark, I. A. *Infect. Immun.* **1991**, *59*, 3280.
10. Cerecetto, H.; González, M. In *Topics in Heterocyclic Chemistry*; Khan, M. T. H., Ed.; Bioactive Heterocycles; Springer: Berlin/Heidelberg, 2007; Vol. IV, pp 265–308. and references therein.
11. Cerecetto, H.; Di Maio, R.; González, M.; Risso, M.; Saenz, P.; Seoane, G.; Denicola, A.; Peluffo, G.; Quijano, C.; Olea-Azar, C. *J. Med. Chem.* **1999**, *42*, 1941.
12. Galli, U.; Lazzarato, L.; Bertinaria, M.; Sorba, G.; Gasco, A.; Parapini, S.; Taramelli, D. *Eur. J. Med. Chem.* **2005**, *40*, 1335.
13. (a) Götz, M. G.; Caffrey, C. R.; Hansell, E.; McKerrow, J. H.; Powers, J. C. *Bioorg. Med. Chem.* **2004**, *12*, 5203; (b) Doyle, P. S.; Zhou, Y. M.; Engel, J. C.; McKerrow, J. H. *Antimicrob. Agents Chemother.* **2007**, *51*, 3932; (c) Jaishankar, P.; Hansell, E.; Zhao, D. M.; Doyle, P. S.; McKerrow, J. H.; Renslo, A. R. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 624.
14. (a) Andrade, S. G.; Stocker-Guerret, S.; Pimentel, A. S.; Grimaud, J. A. *Mem. Inst. Oswaldo Cruz* **1991**, *86*, 187; (b) Bustamante, J. M.; Presti, M. S.; Rivarola, H. W.; Fernández, A. R.; Enders, J. E.; Fretes, R. E.; Paglini-Oliva, P. *Int. J. Antimicrob. Agents* **2007**, *29*, 733; (c) Caldas, S.; Santos, F. M.; de Lana, M.; Diniz, L. F.; Machado-Coelho, G. L.; Veloso, V. M.; Bahia, M. T. *Exp. Parasitol.* **2008**, *118*, 315.
15. (a) Calvino, R.; Mortarini, V.; Gasco, A. *Eur. J. Med. Chem.* **1980**, *15*, 485; (b) Boschi, D.; Sorba, G.; Bertinaria, M.; Fruttero, R.; Calvino, R.; Gasco, A. *J. Chem. Soc. Perkin Trans.* **2001**, *1*, 1751; (c) Sorba, G.; Ermondi, G.; Fruttero, R.; Galli, U.; Gasco, A. *J. Het. Chem.* **1996**, *33*, 327; (d) Bertinaria, M.; Galli, U.; Sorba, G.; Fruttero, R.; Gasco, A.; Brenciaglia, M. I.; Scaltrito, M. M.; Dubini, F. *Drug Dev. Res.* **2003**, *60*, 225; (e) Farrar, W. V. *J. Chem. Soc.* **1964**, 904; (f) Boschi, D.; Di stilo, A.; Cena, C.; Lolli, M.; Fruttero, R.; Gasco, A. *Pharm. Res.* **1997**, *14*, 1750; (g) Medana, C.; Ermondi, G.; Fruttero, R.; Di Stilo, A.; Ferretti, C.; Gasco, A. *J. Med. Chem.* **1994**, *37*, 4412; (h) Sorba, G.; Medana, C.; Fruttero, R.; Cena, C.; Di Stilo, A.; Galli, U.; Gasco, A. *J. Med. Chem.* **1997**, *40*, 463; Sorba, G.; Medana, C.; Fruttero, R.; Cena, C.; Di Stilo, A.; Galli, U.; Gasco, A. *J. Med. Chem.* **1997**, *40*, 2288; (i) Lolli, M. L.; cena, C.; Medana, C.; Lazzarato, L.; Morini, G.; Coruzzi, G.; Manarini, S.; Fruttero, R.; Gasco, A. *J. Med. Chem.* **2001**, *44*, 3463; (j) Calvino, R.; Fruttero, R.; Ghigo, D.; Bosia, A.; Pescarmona, G. P.; Gasco, A. *J. Med. Chem.* **1992**, *35*, 3296; (k) Fruttero, R.; Boschi, D.; Di Stilo, A.; Gasco, A. *J. Med. Chem.* **1995**, *38*, 4944; (l) Fruttero, R.; Ferrarotti, B.; Serafino, A.; Gasco, A. *Liebigs. Ann. Chem.* **1990**, 335; (m) Gagneux, M. *Helv. Chim. Acta* **1970**, *53*, 1883; (n) Calvino, R.; Serafino, A.; Ferrarotti, B.; Gasco, A.; Sanfilippo, A. *Arch. Pharm. (Weinheim)* **1984**, *317*, 695; (o) Fruttero, R.; Boschi, D.; Fornatto, E.; Serafino, A.; Gasco, A.; Exner, O. *J. Chem. Res. Miniprint* **1998**, *9*, 2545; (p) Di Stilo, A.; Cena, C.; Gasco, A. M.; Gasco, A.; Ghigo, D.; Bosia, A. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2607; (q) Gasco, A. M.; Boschi, D.; Di Stilo, A.; Medana, C.; Gasco, A.; Martorana, P. M.; Schonafinger, K. . *Arzneim. Forsch.* **1998**, *48*, 212; (r) Gift of Sanofi-Aventis GmbH.; (s) Pollet, P.; Gellin, S. *Synthesis* **1979**, *12*, 977; (t) Ovchinnikov, I. V.; Kulikov, A. S.; Makhova, N. N.; Tosco, P.; Di Stilo, A.; Fruttero, R.; Gasco, A. *Farmaco* **2003**, *58*, 677; (u) Gasco, A.; Mortarini, V.; Reynaud, E. *Farmaco* **1973**, *28*, 624; (v) Gasco, A. M.; Cena, C.; Di Stilo, A.; Ermondi, G.; Medana, C.; Gasco, A. *Helv. Chim. Acta* **1996**, *79*, 1803; (w) Chegaev, K.; lazarato, L.; Rolando, B.; Marini, E.; Tosco, P.; Cena, C.; Fruttero, R.; Bertolini, F.; Reist, M.; Carrupt, P. A.; Lucini, V.; Fraschini, F.; Gasco, A. *J. Pineal Res.* **2007**, *42*, 371; (x) Barbiere, J. *Bull. Soc. Chim. Fr.* **1944**, *11*, 470; (y) Romanova, L. B.; Ivanova, M. E.; Nesterenko, L. T.; Ermenenko, L. T. *Russ. Chem. Bull.* **1994**, *43*, 1207; (z) Synthetic procedure will be reported elsewhere.
16. (a) Faucher, J. F.; Baltz, T.; Petry, K. G. *Parasitol. Res.* **1995**, *81*, 441; (b) Almeida-de-Faria, M.; Freymuller, E.; Colli, W.; Alves, M. J. *Exp. Parasitol.* **1999**, *92*, 263.
17. (a) Filardi, L. S.; Brener, Z. *Trans. R. Soc. Trop. Med. Hyg.* **1987**, *81*, 755; (b) Molina, J.; Brener, Z.; Romanha, A. J.; Urbina, J. A. *J. Antimicrob. Chem.* **2000**, *46*, 137.
18. Muelas-Serrano, S.; Nogal-Ruiz, J. J.; Gómez-Barrio, A. *Parasitol. Res.* **2000**, *86*, 999.
19. (a) Boiani, M.; Boiani, L.; Denicola, A.; Torres de Ortiz, S.; Serna, E.; Vera de Bilbao, N.; Sanabria, L.; Yaluff, G.; Nakayama, H.; Rojas de Arias, A.; Vega, C.; Rolan, M.; Gómez-Barrio, A.; Cerecetto, H.; González, M. *J. Med. Chem.* **2006**, *49*, 3215; (b) Gerpe, A.; Aguirre, G.; Boiani, L.; Cerecetto, H.; González, M.; Olea-Azar, C.; Rigol, C.; Maya, J. D.; Morello, A.; Piro, O. E.; Arán, V. J.; Azqueta, A.; López de Ceráin, A. L.; Monge, A.; Rojas, M. A.; Yaluff, G. *Bioorg. Med. Chem.* **2006**, *14*, 3467.
20. (a) Sereno, D.; Lemesre, J.-L. *Parasitol. Res.* **1997**, *83*, 401; (b) Dutta, A.; Bandyopadhyay, S.; Mandal, C.; Chatterjee, M. *Parasitol. Int.* **2005**, *54*, 119.
21. Maarouf, M.; De Kouchkovsky, Y.; Brown, S.; Petit, P. X.; Robert-Gero, M. *Exp. Cell Res.* **1997**, *232*, 339.
22. (a) Mesa-Valle, C. M.; Castilla-Calvente, J.; Sanchez-Moreno, M.; Moraleda-Lindez, V.; Barbe, J.; Osuna, A. *Antimicrob. Agents Chemother.* **1996**, *40*, 684; (b) Sánchez-Moreno, M.; Fernández-Becerra, C.; Castilla, J.; Osuna, A. *FEMS Microbiol. Lett.* **1995**, *133*, 119; (c) Fernandez-Ramos, C.; Luque, F.; Fernández-Becerra, C.; Osuna, A.; Jankevicius, S. I.; Jankevicius, V.; Rosales, M. J.; Sanchez-Moreno, M. *FEMS Microbiol. Lett.* **1999**, *170*, 343.
23. Caligiani, A.; Acquotti, D.; Palla, G.; Bocchi, V. *Anal. Chim. Acta* **2007**, *585*, 110.
24. Penin, P.; Sánchez-Moreno, M.; de Diego, J. A. *Comp. Biochem. Physiol.* **1998**, *120A*, 571.
25. Cannata, J. J. B.; Cazzulo, J. J. *Comp. Biochem. Physiol.* **1984**, *79B*, . 297.
26. (a) Tielens, A. G. M.; Van Hellemond, J. J. *Parasitol. Today* **1998**, *14*, 265; (b) Coustou, V.; Besteiro, S.; Riviere, L.; Biran, M.; Biteau, N.; Franconi, J-M.; Boshart, M.; Baltz, T.; Bringaud, F. *J. Biol. Chem.* **2005**, *280*, 16559; (c) Takashima, E.; Inaoka, D. K.; Osanai, A.; Nara, T.; Odaka, M.; Aoki, T.; Inaka, K.; Harada, S.; Kita, K. *Mol. Biochem. Parasitol.* **2002**, *122*, 189.
27. (a) Aguirre, G.; Cerecetto, H.; Di Maio, R.; González, M.; Seoane, G.; Denicola, A.; Ortega, M. A.; Aldana, I.; Monge, A. *Arch. Pharm.* **2002**, *335*, 15; (b) Aguirre, G.; Boiani, M.; Cerecetto, H.; Gerpe, A.; González, M.; Fernández, Y.; Denicola, A.; Ochoa, C.; Nogal, J. J.; Montero, D.; Escario, J. A. *Arch. Pharm.* **2004**, *337*, 259; (c) Aguirre, G.; Boiani, L.; Boiani, M.; Cerecetto, H.; Di Maio, R.; González, M.; Porcal, W.; Denicola, A.; Piro, O. E.; Castellano, E. E.; Sant'Anna, M.; Barreiro, E. J. *Bioorg. Med. Chem.* **2005**, *13*, 6336; (d) Caterina, M. C.; Perillo, I. A.; Boiani, L.; Pezaroglo, H.; Cerecetto, H.; González, M.; Salerno, A. *Bioorg. Med. Chem.* **2008**, *16*, 2226.
28. Bernacchi, A. S.; Franke de Cazzulo, B.; Castro, J. A.; Cazzulo, J. J. *Acta Pharmacol. Sin.* **2002**, *23*, 399.