

Arylfurans as potential *Trypanosoma cruzi* trypanothione reductase inhibitors

Renata B de Oliveira, Aline BM Vaz, Rosana O Alves*, Daniel B Liarte*, Claudio L Donnici**, Alvaro J Romanha*, Carlos L Zani/+

Laboratório de Química de Produtos Naturais *Laboratório de Parasitologia Celular e Molecular, Centro de Pesquisas René Rachou-Fiocruz, Av. Augusto de Lima 1715, 30190-002 Belo Horizonte, MG, Brasil **NEQUIM - Departamento de Química, Instituto de Ciências Exatas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil

The natural lignans veraguensin and grandisin have been reported to be active against *Trypanosoma cruzi* bloodstream forms. Aiming at the total synthesis of these and related compounds, we prepared three 2-arylfurans and eight 2,5-diarylfurans. They were evaluated for their potential as *T. cruzi* trypanothione reductase (TR) inhibitors as well against the parasite's intracellular (amastigote) and bloodstream (trypomastigote) forms. Compound **12** was the most effective against TR with an IC_{50} of 48.5 μ M while **7** and **14** were active against amastigotes, inhibiting the parasite development by 60% at 20 μ g/ml (59 and 90 μ M, respectively). On the other hand, none of the compounds was significantly active against the parasite bloodstream forms even at 250 μ g/ml (0.6-1.5 mM).

Key words: tropical diseases - Chagas disease - arylfurans - trypanothione reductase

Chagas disease, caused by the flagellate protozoan *Trypanosoma cruzi*, affects 18 million people in Latin America and is responsible for 13,000 deaths every year (WHO 2002). The treatment relies on only two available drugs, nifurtimox and benznidazole, which are relatively efficient in the acute phase of the disease, but almost ineffective in the chronic phase. Nowadays, one of the most important mechanisms of Chagas disease transmission in many countries is by blood transfusion (Schmuñis 1991). In highly endemic areas it is strongly recommended the use of chemoprophylactic measures such as the addition of gentian violet to clear trypomastigotes from blood banked for transfusion (Moraes-Souza et al. 1995). Although effective, this triphenylmethane dye is not well accepted because of undesirable effects such as coloring the skin and possible mutagenicity (Wendel 1993). Thus, new drugs to treat or prevent Chagas disease are still needed.

Trypanosoma cruzi enzymes such as the trypanothione reductase (TR) represent a potential drug targets because they play an essential role in the life of this parasite. TR and its substrate trypanothione, the disulfide of a glutathione-spermidine conjugate [N^1 , N^8 -bis(glutathionyl)spermidine, $T(SH)_2$] **1**, help to protect the parasite from oxidative stress by maintaining an intracellular reducing environment in a manner analogous to glutathione reductase (GR) and glutathione [L- γ -glutamyl-L-cysteinylglycine, GSH] **2** (Fig. 1a) in mammalian cells (Schmidt & Krauth-Siegel 2002). TR catalyses the NADPH-dependent reduction of trypanothione disulfide TS_2 to its

dithiol form, $T(SH)_2$. Trypanothione may be oxidized back to TS_2 (Fig. 1b) following reaction with potentially damaging radicals and oxidants generated by aerobic metabolism. Another aspect that makes TR an even more attractive target is its structural differences from the human counterpart GR. GR has a narrow positively charged active site, to accommodate the glycine carboxylates of its substrate glutathione, whereas TR has a wider, non-charged, and more hydrophobic active site (Bond et al. 1999). These differences allowed the discovery of several promising selective inhibitors of TR (Schmidt & Krauth-Siegel 2002).

Lignans is a class of natural products that possess important biological properties (Jensen et al. 1993). Lopes et al. (1998) showed that the tetrahydrofuran lignans veraguensin **3** and grandisin **4** (Fig. 2) were active in vitro at 5 μ g/ml against the trypomastigote of *T. cruzi* present in murine blood, causing 100% of parasite lysis without damaging erythrocytes. The activity of these lignans was

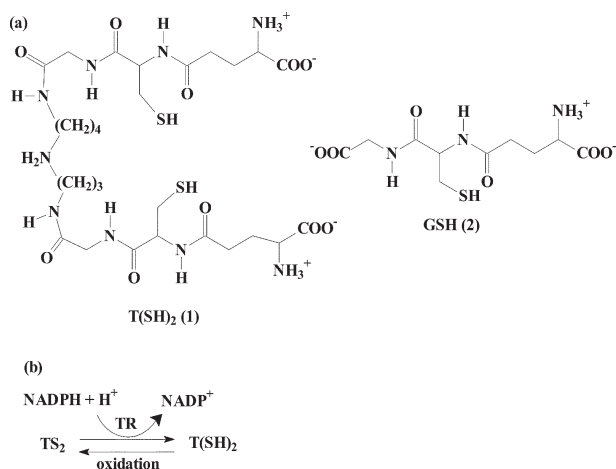


Fig.1: (a) chemical structure for trypanosomal trypanothione **1** and human glutathione **2**; (b) trypanothione reductase (TR)-catalysed reduction of TS_2 .

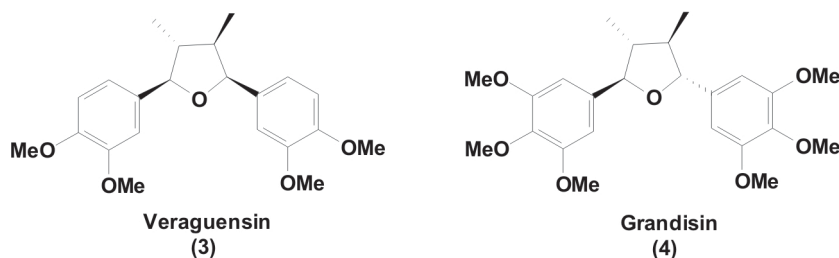


Fig. 2: lignans with trypanocidal activity isolated from *Virola surinamensis* (Lopes et al. 1998).

fifty times higher than that of the reference drug gentian violet.

Based on these promising results, we decided to synthesize these natural products and some analogues to evaluate their activity against *T. cruzi*. Our synthetic route to **3** and **4** (Fig. 3) involved arylfurans as intermediates.

Arylfurans present a broad-spectrum of biological activities [for example, antimicrobial activity (Stephens et al. 2001, Lanteri et al. 2004), effects against neurodegenerative, cardiovascular, and proliferative diseases (Lockhart et al. 2004), inhibitory activity against the enzymes farnesyltransferase (Mitsch et al. 2004) and PDE4 (Perrier et al. 1999), vascularization inhibitor effect (Kuвано et al. 1994)] and previous works (Jockers-Scherübl et al. 1989, Paulino et al. 2002, Aguirre et al. 2004) have shown that furan derivatives could be potential ligands for TR. In view of these results, and aiming at the discovery of new trypanocidal compounds we evaluated the effect of the synthetic compounds **5-15** (Fig. 4) on TR and the whole trypomastigote and amastigote forms of the parasite.

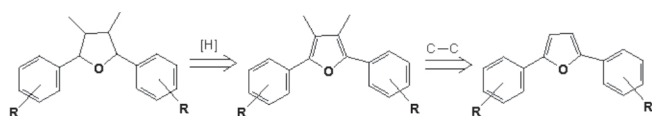


Fig. 3: arylfuran analogues of lignans **3** and **4**.

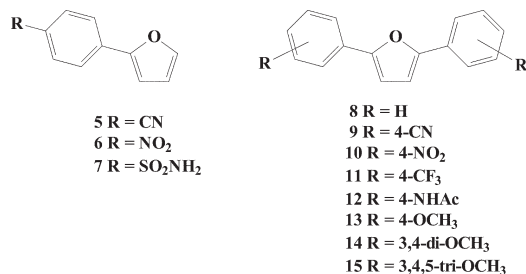


Fig. 4: chemical structures of 2-arylfurans (**5-7**) and 2,5-diarylfurans (**8-15**)

MATERIALS AND METHODS

Chemistry - The 2-arylfurans **5-7** and the 2,5-Bis(*p*-cyanophenyl)furan **9** were synthesized in one step using the classical Meerwein arylation (treatment of furan with diazonium salts in the presence of cupric salts) (Rondestedt 1976). The 2,5-diarylfurans **8, 10-15** were prepared in two steps: (1) preparation of 2,5-bis

(trimethylstannyl)furan **16** by reaction of furan with TMEDA and *n*-butyllithium and subsequent addition of trimethyltin chloride (Seitz et al. 1983), and (2) palladium catalyzed coupling reaction between the distannane **16** and various arylhalides (Stille coupling) (Stephens et al. 2001).

In vitro assay with *T. cruzi* TR - Recombinant *T. cruzi* TR was obtained as described by Borges et al. (1995). The colorimetric microtitre plate assay was adapted from that described by Hamilton et al. (2003). It was run in 40 mM HEPES (pH 7.5), 1 mM EDTA, 0.12 mM NADPH and 0.8 μM trypanothione TS₂ in a total assay volume of 250 μl. The test compounds were dissolved in DMSO and diluted with water to a final concentration of 0.1% (v/v) DMSO. After addition of the enzyme (5.12 mU/well) to the compound solution (20 μg/ml, 50-120 μM) the mixture was incubated for 30 min at 30°C, after which 25 μM of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was added and the absorbance at 410 nm measured in a kinetic mode for 5 min. Clomipramine at its IC₅₀ (6.5 μM) was used as positive and 0.1% (v/v) DMSO as negative controls. Each compound was tested in triplicate. The results are expressed as percentage reduction of the TR activity. For IC₅₀ determinations, inhibition assays were carried out at ten different concentrations and repeated four times.

In vitro assay with *T. cruzi* blood stream forms - The assays with *T. cruzi* were carried out using blood from Swiss albino mice collected in the parasitaemia peak (7th day) after infection with the Y strain of *T. cruzi*. The infected blood was diluted with normal murine blood and RPMI 1640 medium 1:2 (pH 7.2-7.4) to the concentration of 2 × 10⁶ trypomastigotes/ml. Stock solutions at 10 mg/ml (25-59 mM) of the compounds were prepared in dimethylsulfoxide (DMSO). A sample (5.0 μl) of each solution was added to 195 μl of infected blood providing a final concentration of 250 μg/ml (0.6-1.5 mM). Samples of 100 μl were transferred in duplicate to the wells of a microtitre plate (96 wells). To reproduce the blood bank conditions, plates were incubated at 4°C for 24 h. The experiments were repeated two times. Afterwards, the parasite concentration was evaluated using an optical microscope with a 400 X magnification. DMSO at 2.5% v/v and gentian violet at its IC₅₀ concentration (7.5 μg/ml) were used as negative and positive controls, respectively. DMSO was not added in the positive control. The parasite concentration reduction (parasite lysis) was determined in comparison with negative control containing only 2.5% DMSO. At 2.5% concentration DMSO in blood was found to cause no morphological alterations or lysis either in the parasites, erythrocytes or leukocytes.

In vitro assay with *T. cruzi* amastigote forms (Buckner et al. 1996 modified) - Parasites and culture procedures: *T. cruzi* (Tulahuen strain) expressing the *Escherichia coli* beta-galactosidase gene were grown on monolayer of mouse L929 fibroblasts. Cultures to be assayed for beta-galactosidase activity were grown in RPMI 1640 medium (pH 7.2-7.4) without phenol red (Gibco BRL) plus 10% fetal bovine serum, glutamine and gentamicin.

T. cruzi growth inhibition assay - Ninety-six-well tissue culture plates were seeded with L929 fibroblasts at 4.0×10^3 per well in 80 μ l and incubated overnight. Beta-galactosidase-expressing trypomastigotes were then added at 4.0×10^4 per well in 20 μ l. After 2 h, the medium with trypomastigotes that not penetrated in cells was discarded and replaced by 200 μ l of fresh medium. After 48 h, the medium was discarded again and replaced by 180 μ l of fresh medium and test compounds in 20 μ l. Each compound was tested in triplicate. After 7 days of incubation, chlorophenol red beta-D-galactopyranoside (CPRG) (100 mM final concentration) and Nonidet P-40 (0.1% final concentration) were added to the plates and incubated overnight at 37°C and the absorbance measured at 570 nm in an automated micro plate reader. Benznidazole at its IC_{50} (4.0 μ M) was used as positive control. The results are expressed as percentage growth inhibition.

RESULTS AND DISCUSSION

The monoarylfurans **5-7**, and the diarylfurans **9** were synthesized in a single step *via* the Meerwein arylation in moderate yields. Initial attempts to prepare 2,5-diarylfurans by this method were unsuccessful and only mono-arylfurans were formed, except for compound **9**, obtained with 12% yield. Under this reaction condition anilines containing electron donor substituents attached to the phenyl ring furnished only tarry material from which nei-

ther mono nor diarylfurans could be isolated. Therefore, the 2,5-diarylfurans **8**, **10-15** were prepared in two steps using the Stille coupling. The structures of all compounds were confirmed by spectroscopy and physicochemical data (Table I).

Table II shows the results of the bioassays using the pure compounds **5-15** against TR and *T. cruzi* trypomastigote and amastigote forms. For the comparison of the activities standard control drugs were included in the assays.

The compounds were tested for inhibition of TR at 20 μ g/ml and the IC_{50} values were determined for the most active compounds. Thus, the nitro derivative **6** and the diacetamide **12** presented IC_{50} values of 155 μ M and 48.5 μ M, respectively. Many nitrofuran derivatives have been reported to act as subversive substrate for TR, a class of inhibitors that produce free radicals once reduced by the enzyme, thereby subverting its physiological role (Jockers-Scherübl et al. 1989, Paulino et al. 2002, Maya et al. 2003, Chibale & Musonda 2003, Aguirre et al. 2004). In the presence of oxygen, these inhibitors are cyclically reduced and reoxidized generating deleterious oxygen radicals while simultaneously inhibiting TR's ability to reduce its physiological substrate (Chibale & Musonda 2003). Thus, the inhibitory activity presented by the nitro derivative **6** could be associated to its ability to act as subversive substrates. On the other hand the related diarylfuran **10**, despite containing two NO_2 groups, was less active than **6**, probably due to its poor solubility in the assay medium. The diacetamide **12** was the best inhibitor and its activity may come from to the combination of the hydrophobic moiety with the amide groups. The amide groups may be important for the interaction between compound **12** and the enzyme *via* hydrogen bonds. However, further experimental evidences are needed to confirm such possibility.

TABLE I
Spectroscopic and physicochemical data for arylfurans

Compd	Yield(%)	mp (obs.) (°C)	mp (lit.) (°C)	MS(<i>m/z</i>)	¹ H NMR 200 MHz δ (ppm)
5	24	64.3-65.8	61-63 ^a	169, 88, 70, 61	6.52 (dd, 1H); 7.81 (dd, 1H); 7.53 (dd, 1H); 7.63 (d, 2H); 7.73 (d, 2H)
6	48	133.9-134.4	134-135 ^b	189, 159, 115, 63	6.55 (dd, 1H); 6.87 (d, 1H); 7.57 (d, 1H); 7.78 (d, 2H); 8.24 (d, 2H)
7	30	243.4-244.2	-	223, 03130 ^c	6.65 (dd, 1H); 7.13 (d, 1H); 7.39 (sl, 2H); 7.82 (d, 1H); 7.87 (m, 4H)
8	40	86.1-87.3	87 ^d	220, 105, 77, 61	6.74 (s, 2H); 7.23-7.78 (m, 10H)
9	12	291.4-293.1	294-295 ^e	270, 69, 55, 41	6.96 (s, 2H); 7.71 (d, 4H); 7.83 (d, 4H)
10	72	269.8-271.2	269-270 ^f	310, 280, 234, 189	7.54 (s, 2H); 8.13 (d, 4H); 8.32 (d, 4H)
11	34	143.9-145.7	-	356, 337, 183, 145	6.88 (s, 2H); 7.67 (d, 4H); 7.84 (d, 4H)
12	21	307-309	307-308 ^g	334, 292, 250, 207	2.06 (s, 6H); 6.92 (s, 2H); 7.65 (d, 4H); 7.72 (d, 4H); 10.05 (s, 2H)
13	35	189-190	195-196 ^h	280, 265, 135	3.86 (s, 6H); 6.59 (s, 2H); 6.95 (d, 4H); 6.67 (d, 4H)
14	26	158.6-158.9	154-155 ⁱ	340, 325, 170, 44	3.94 (s, 6H); 4.00 (s, 6H); 6.63 (s, 2H); 6.93 (d, 2H); 7.26 (sl, 2H); 7.33 (d, 2H)
15^j	40	140-140.9	-	400, 385, 60	3.89 (s, 6H); 3.95 (s, 12H); 6.67 (s, 2H); 6.95 (s, 4H)

a: Ohta et al. 1990; b: Fisera et al 1974; c: Calcd. for $C_{10}H_9NO_3S$ (223,03031); d: Koga et al 1998; e: Das & Boykin 1977; f: Stephens et al 2001; g: Jonh & Robert 1958; h: Bailey et al 1965; i: Haworth & Kelly 1937; j: Anal. Calcd. for $C_{22}H_{24}O_7$: C 65.99, H 6.04. Found: C 65.64, H 5.57.

TABLE II
Trypanothione reductase (TR) inhibition and trypanocidal activity of arylfurans

Compound	TR inhibition		Trypanocidal activity		Log P ^a
	Inhibition at 20 µg/ml (%)	IC ₅₀ (µM)	Blood trypomastigotes (% lysis at 250 µg/ml)	Intracellular amastigotes (% growth inhibition at 20 µg/ml)	
Clomipramine	-	6.5	-	72	-
Crystal violet	-	-	53 ^b	-	-
Benznidazole	-	-	-	50 ^c	-
5	5	-	12	35	2.72
6	34	155	16	30	2.63
7	8	-	8	70	1.57
8	4	-	21	25	4.13
9	11	-	3	35	4.20
10	9	-	2	25	4.04
11	10	-	13	28	5.89
12	54	48.5	5	15	0.39
13	15	-	3	22	3.62
14	7	-	6	65	3.24
15	3	-	41	9	5.45

a: calcd. using the HyperChem 6.02 Program software; b: crystal violet was tested at 3.0 µM; c: benznidazole was tested at 4.0 µM.

The results in Table II show no correlation between enzyme inhibition and trypanocidal activity as the two major TR inhibitors, compounds **6** and **12**, showed no effect against intact bloodstream or amastigote forms of the parasite. On the other hand, compound **15**, inactive against TR, reduced by 41% the number of trypomastigotes in the blood while compounds **7** and **14** were able to inhibit by more than 60% the growth of intracellular amastigotes. Interestingly compound **14** is structurally related with the lignan veraguensin **3**. Table II shows the calculated partition coefficients for the arylfurans **5-15**. Partition coefficient between water and octanol (log P) is regarded as a measure of lipophilicity of a drug and is related to its ability to cross biological membranes. Substances with high log P values dissolve better in fats and oils than in water. This enhances their ability to enter lipid membranes by passive diffusion, thereby enhancing their potential for absorption. Generally, for a good activity the relationship appears to be parabolic with an optimum Log P value of around 2 ± 1 . It is well known that compounds with negative log P values cross cell membranes very poorly (Lipinski et al. 1997). The log P values for compounds **11** and **15** are outside the optimum limits. Compound **6** has a reasonable log P value but its IC₅₀ is so high that an effective concentration inside the parasite would be difficult to reach. The low trypanocidal activity of the diamide **12** may be due to factors such as a) poor membrane permeability as indicated by its low log P value (log P = 0.39); b) the in vitro assay with *T. cruzi* bloodstream forms mimics blood bank conditions and is carried in a short assay time (24 h) and low temperature (4°C), making it difficult to detect the interference of the compound on the TR activity; c) In the intracellular assay with amastigotes the compound **12** could not be able to cross the fibroblast and parasite's cell membranes to reach

parasite's cytoplasm in sufficient quantity to significantly inhibit TR. Therefore, a direct correlation between the trypanocidal activity and inhibition of TR by the compounds was not observed. Indeed, even clomipramine, one of the most potent inhibitor of TR presented only moderate activity (72% inhibition) against intracellular amastigote forms at 57 µM (20 µg/ml).

Despite its failure to reduce parasites under the conditions used in the present work, compound **12** deserves further investigation to determine its TR inhibition mechanism and to develop related compounds with improved potency.

ACKNOWLEDGMENTS

To Prof. Alan Fairlamb, University of Dundee, Scotland, for providing the recombinant *T. cruzi* TR and to Prof. Frederick S Buckner, University of Washington, for providing the Tulahuen strain of *T. cruzi* expressing the *E. coli* beta-galactosidase gene used in this work.

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