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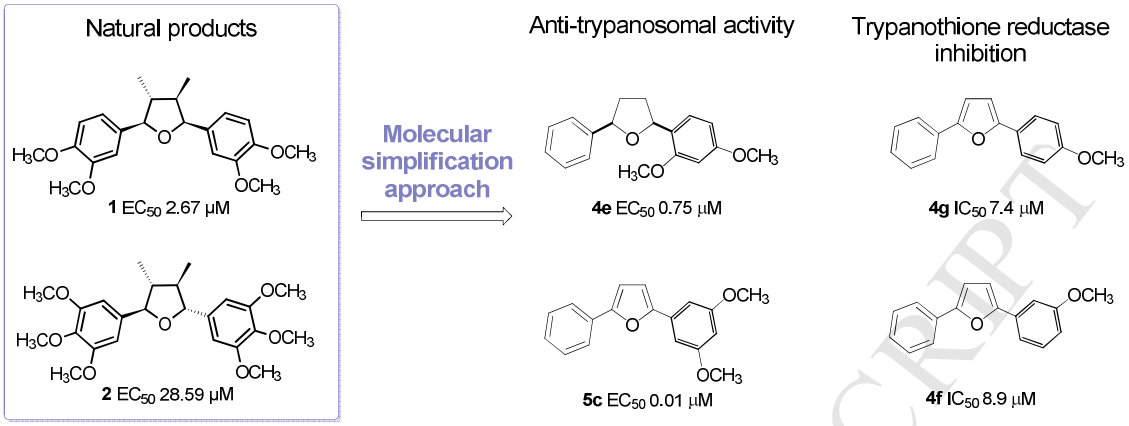
Graphical Abstract



Synthesis and 2D-QSAR studies of neolignan-based diaryl-tetrahydrofuran and -furan analogues with remarkable activity against *Trypanosoma cruzi* and assessment of the trypanothione reductase activity

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Abstract

Two series of diaryl-tetrahydrofuran and -furan were synthesised and screened for anti-trypanosomal activity against trypomastigote and amastigote forms of *Trypanosoma cruzi*, the causative agent of Chagas disease. Based on evidence that modification of a natural product may result in a more effective drug than the natural product itself, and using known neolignan inhibitors veraguensin **1** and grandisin **2** as templates to synthesise simpler analogues, remarkable anti-trypanosomal activity and selectivity were found for 3,5-dimethoxylated diaryl-tetrahydrofuran **5c** and 2,4-dimethoxylated diarylfuran **4e** analogues with EC_{50} 0.01 µM and EC_{50} 0.75 µM, respectively, the former being 260-fold more potent than veraguensin **1** and 150-fold better than benznidazole, the current available drugs for Chagas disease treatment. The ability of the most potent anti-trypanosomal compounds to penetrate LLC-MK2 cells infected with *T. cruzi* amastigotes parasite was tested, which revealed **4e** and **5e** analogues as the most effective, causing no damage to mammalian cells. In particular, the majority of the derivatives were non-toxic against mice spleen cells. 2D-QSAR studies show the rigid central

core and the position of dimethoxy-aryl substituents dramatically affect the anti-trypanosomal activity. The mode of action of the most active anti-trypanosomal derivatives was investigated by exploring the anti-oxidant functions of Trypanothione reductase (TR). As a result, diarylfuran series displayed the strongest inhibition, highlighting compounds **5d-e** (IC_{50} 19.2 and 17.7 μM) and **5f-g** (IC_{50} 8.9 and 7.4 μM), respectively, with similar or 2-fold higher than the reference inhibitor clomipramine (IC_{50} 15.2 μM).

Keywords: *Trypanosoma cruzi*, Chagas disease, natural neolignans, diaryl-tetrahydrofurans, diarylfurans, trypanothione reductase, 2D-QSAR.

2. Introduction

Plant-derived natural products represent a valuable source of novel drug leads because of the chemical diversity found in nature, giving rise to highly active compounds with selective and specific mechanisms of action [1,2]. Despite the great interest of pharmaceutical companies in combinatorial chemistry to access the synthetic compound library for HTS (High Throughput Screen) approaches, the decline of new chemical entities approved by the FDA at the beginning of the 21st century, has led the research community to reappraise plant-derived natural product drug discovery as an important approach for the identification of novel chemotherapeutics agents [3]. Furthermore, the extensive use of natural products by different cultures for the treatment of various disease conditions make them a valuable supply of new drugs [1,4,5].

Apart from that, several aspects preclude the successful research, development and commercialisation of natural products, such as the supply constraints on bioactive components, ecological and legal considerations for plant accessibility, the isolation processes, variation of quality and composition (depend on species identity), harvest time, soil composition, altitude, actual climate, and processing and storage conditions [3,6]. Even though the resupply of natural products can be achieved by the application of plant cell and tissue culture, heterologous production or semi-synthesis from isolated precursors. The limited amount of isolated bioactive natural products available, and the total chemical synthesis of complex structures containing several functional groups and chiral centers, slow down the development process, which still requires optimisation of physicochemical properties using chemical modification approaches [3,7]. Therefore, the chemical simplification and derivatisation of natural products is an interesting alternative to generate a chemical library, as

well as to understand the structure–activity relationship for pharmacophore identification. In the last decade, several natural product extracts have been screened to find more active and less toxic molecules against Chagas disease, a life-threatening illness caused by the protozoan parasite *Trypanosoma cruzi* [8-10]. Chagas disease is endemic in Central and South American countries, with an estimated 8 million people infected worldwide [11,12]. In 2011, Nakamura reported 400 species related to approximately 100 families of plant extracts already tested against different forms of *T. cruzi*, of which 10% were investigated and their corresponding active components against the parasite were isolated and characterised, being around 136 compounds assessed [13]. Since then, a brief search at Web of Science [14] revealed about 33 active compounds from plant extracts against *T. cruzi* [15-26]. The phenotypic screening of potential anti-trypanosomal compounds from natural products has shown a plethora of chemical classes, illustrated by alkaloid, flavonoid, catechin and terpene derivatives, including sesquiterpene lactones, xanthone, oxygenated hydrocarbons and lignans [13,27,28]. In particular, lignans isolated from Lauraceae and Piperaceae families are of great interest since the majority of the active components decrease parasite growth of both epimastigote and trypomastigote forms [29]. Beside lignans, tetrahydrofuran neolignans such as veraguensin (**1**) and grandisin (**2**), isolated from *Virola surinamensis* and *Piper solmsianum*, have been identified as potent inhibitors of trypomastigote Y strain of *T. cruzi*, producing parasite lysis with EC_{50} 2.3 and 3.7 µM, respectively [30] (Figure 1). Notably, veraguensin (**1**) and grandisin (**2**) possess 2,5-bis(3,4-dimethoxyphenyl)- and 2,5-bis(3,4,5-trimethoxyphenyl)-tetrahydro-3,4-dimethylfuran structures, respectively. In addition, 4-(furan-2-yl) benzenesulfonamide and symmetrical non-substituted 2,5-bis(3,4-dimethoxyphenyl)furan have been further described as *T. cruzi* amastigote growth inhibitors (70 and 65% inhibition at 20 µg/mL) [31]. Our previous studies regarding the low water solubility of these neolignans have shown the importance of the heterocycle core to the potency [32,33], considering the distinguished activity displayed by dihydrofuran derivative **3**, the most active of all series with EC_{50} 1.5 µM [32], when comparing the activities of **1-3** with open chain symmetrical and unsymmetrical diaryl diols and diaryl diketones precursors, besides symmetrical acetylenic glycols, which preserve the diaryl polymethoxylated pattern of natural products **1** and **2**. These findings encouraged us to investigate whether simplified unsymmetrical 2,5-diaryl-tetrahydrofuran and -furan derivatives containing methoxyl substituents in a variable number and at different ring positions, such as the set compounds **4** and **5**, would possess enhanced activity against trypomastigote and amastigote forms of *T. cruzi*. Further, 2D-QSAR studies were carried out to identify the molecular descriptors that may encode structure information of the set molecules in order to

predict the anti-parasitic responses of new hit derivatives [34-36]. Then, the most active anti-trypanosomal derivatives were chosen to investigate whether they inhibit Trypanothione reductase (TR), a key enzyme involved in the parasite redox metabolic pathways.

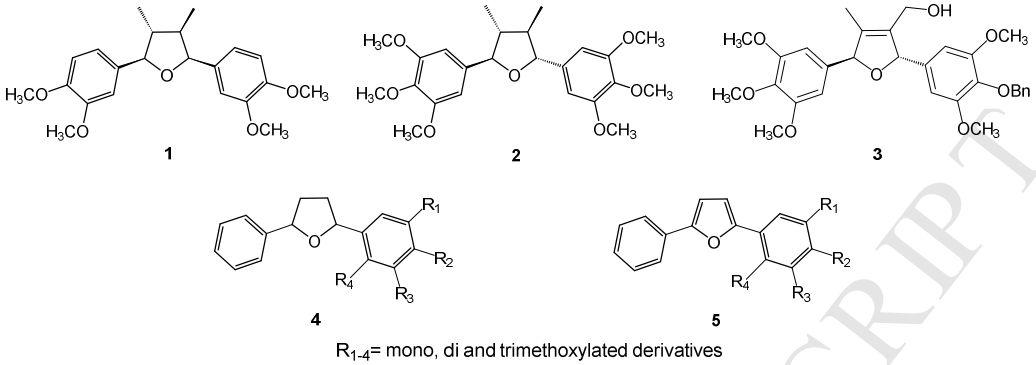


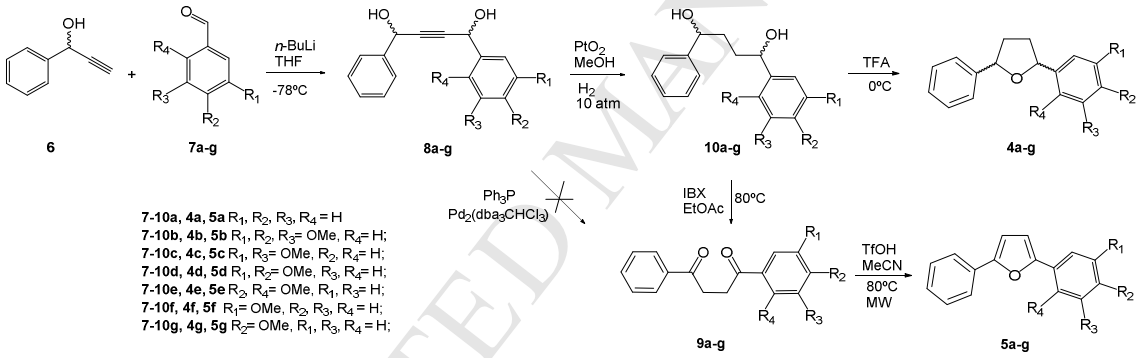
Figure 1. Neolignans veraguensin (**1**) and grandisin (**2**), the synthetic derivative **3** previously described and target simplified structures of neolignan-based diaryl-tetrahydrofuran and -furan analogues (**4**, **5**).

2. Results and Discussion

2.1 Chemistry

As shown in scheme 1, the condensation of the dianion of racemic 1-phenylprop-2-yn-1-ol (**6**) with a diverse set of commercially available aryl aldehydes **7a-g** bearing methoxy groups at different positions of the aromatic ring afforded key intermediates 1,4-diphenyl-2-butyn-1,4-diol (**8a-g**) in good to moderate yields (30-82%) (Scheme 1) [33,37]. Several attempts to improve the yields led to the protection of the hydroxyl group of precursor carbinol **6** to prevent the dianion formation under basic conditions. Typically, the treatment of **6** with *tert*-butyldimethylsilyl chloride promoted the protection of both hydroxyl and acetylenic groups, whereas dihydropyran (DHP) under acid conditions [38] gave the mono-*O*-protected intermediate in 45% yield. Despite the low to moderate yields obtained during the condensation reaction between the *O*-DHP carbinol derivative **6** with a panel of aldehydes **7a-g**, the removal of the *O*-DHP protecting group using several protocols (*p*-TsOH, Dowex®50WX4-50, trifluoro-acetic and acetic acids and 1M HCl) [39-42], proved to be difficult. Therefore, we performed the condensation reactions with unprotected carbinol **6** for formation of the diastereoisomeric mixtures of **8a-g**, which were assessed by ^{1}H NMR analysis with two characteristic singlets or doublets around 5.50 ppm, related to the hydrogen attached to the carbon-linked hydroxyl group (except for the symmetrical intermediate **8a**). Furthermore, ^{13}C NMR revealed clearly the duplicity of signals at 85.0 ppm related to acetylene carbons.

Subsequent one-pot isomerisation of 1,4-diphenyl-2-butyn-1,4-diol (**8a-g**) intermediates with palladium complex $Pd_2(dba_3CHCl_3)$ [tris(dibenzylideneacetone)dipalladium] and triphenylphosphine [43] was not convenient to afford the corresponding diaryl-diketone derivative **9a-g**, since the yields were very low (6%) and time-consuming (70 h). On the other hand, an appropriate two-step procedure, involving the alkyne reduction of **8a-g** intermediates followed by hydroxyl group oxidation, was accomplished to produce both 1,4-diphenylbutane-1,4-diol derivatives **10a-g** and 1,4-diphenylbutane-1,4-dione **9a-g** intermediates, crucial for the generation of target diaryl-tetrahydrofurans **4a-g** and -furans **5a-g**, respectively. As a result, alkyne reduction with platinum oxide at 10 atm [44] proved suitable for formation of **10a** in 92% yield, whilst hydroxyl oxidation with IBX (2-iodoxybenzoic acid) [45] led to the diaryl-diketone **9a** formation in 98% yield, superior to the reaction initially performed with PCC (pyridinium chlorochromate, 49%) [46]. Therefore, the conditions established for sequential conversion of **8a** into compounds **10a** and **9a** were successfully applied to produce the remaining set of compounds.



Scheme 1. Synthesis of 2,5-diaryl-tetrahydrofuran **4a-g** and -furan **5a-g** derivatives, comprising methoxyl substituents at different positions of the aromatic ring, starting from the condensation reaction of the racemic 1-phenylprop-2-yn-1-ol (**6**) with a diverse set of commercially available aryl aldehydes **7a-g**.

Owing to their significant biological activities [47] and natural occurrence [29,30], tetrahydrofuran syntheses have been addressed by a wide variety of approaches, the intramolecular S_N2 reaction between a hydroxyl group and a leaving group being a convenient and practical strategy to afford functionalised cyclic ethers [48]. Therefore, a straightforward synthesis of a mixture of an equal amount of *cis*- and *trans*-2,5-diphenyl-tetrahydrofurans **4a-g** was achieved by cyclisation/dehydration reaction of the diaryl-dihydroxyl derivatives **10a-g**, according to a well-established protocol using trifluoroacetic acid in chloroform [49]. Apart

from the known compounds **4a** [50-55], **4d** [56-57] and **4g** [51,58-60], obtained by different procedures, the novel diastereomers were isolated by column chromatography as a single spot in moderate yield (30–65%) and assigned based on ^{1}H NMR spectra, which showed characteristic overlapping signals at 1.80–2.50 and 4.80–5.20 ppm related, respectively, to H-3/H-4 and H-2/H-5 tetrahydrofuran stereoisomers. Additionally, the ^{13}C NMR also revealed the tetrahydrofuran methylene and methine carbons at 35.0 and 80.0 ppm.

Despite the availability of several metal-catalysed furan procedures [61], the synthesis of 2,5-diphenylfurans **5a-g** was achieved by microwave-assisted Pal-Knorr classical reaction[62] via cyclisation of 1,4-diphenylbutane-1,4-dione intermediates **9a-g** using triflic acid in 20 min, rather than 68 h previously described [63]. The target furan structures **5c-e** were confirmed by ^{1}H and ^{13}C NMR, which showed the signals of the furan ring at 6.30–6.80 ppm and 104.0–107.0/150.0 ppm, respectively, and the NMR spectra of the known compounds **5a** [64,65], **5b** [66], **5f** [65] and **5g** [66] were in accordance with the literature.

After preparing 2,5-diaryl-tetrahydrofuran **4a-g** and -furan derivatives **5a-g**, we next evaluated their anti-trypanosomal activity on trypomastigote forms of Tulahuen using colorimetric assay as a preliminary screening [67]. Thereafter, we assessed the anti-trypanosomal activity of the most potent compounds against the infective Y parasite strain, besides their ability to penetrate the host LLC-MK2 cells and kill the intracellular and replicative *T. cruzi* amastigote form [68,69]. The natural compounds veraguensin (**1**) and grandisin (**2**) and the drug benznidazole, currently used to treat Chagas disease, were also assessed for comparative analysis.

2.2 Anti-trypanosomal activity and cytotoxicity

Trypomastigote forms of *T. cruzi* Tulahuen strain incubated with monkey kidney cells were used to measure parasite viability in the presence of the synthesised compounds (concentrations ranging from 3.75 to 500 µM). Compound concentrations corresponding to 50% anti-trypanosomal activity were calculated based on dose response curves and expressed as EC_{50}. Furthermore, cytotoxicity assays were performed in mice spleen cells and the synthesised compounds were tested at concentrations from 1.50 to 250 µM, using Tween 80 as cell death control. The anti-trypanosomal and cytotoxic activities were compiled in Table 1, besides the selectivity index (SI).

According to Table 1, in the 2,5-diaryl tetrahydrofuran **4a-g** series, presenting closer similarity to veraguensin (**1**) and grandisin (**2**), remarkable anti-trypanosomal activity on

Tulahuen strain and selectivity was achieved by derivative **4e** (2,4-OMe) with EC_{50} 0.75 μM, about twice as high as the activity showed by benznidazole (EC_{50} 1.65 μM). The activity of **4e,** up to 50-fold more potent than the corresponding unsubstituted **4a** (EC_{50} 37.31 μM), revealed a favourable effect of the 2,4-dimethoxy substitution pattern on the aromatic ring in improving anti-trypanosomal activity. The efficacy also displayed by compound **4f** (3-OMe) (EC_{50} 2.29 μM) and the ability of this series to not damage host cells make compounds **4e** and **4f** very promising since they fulfill the TDR criteria for antichagasic agents, based on EC_{50} of <4.0 μM and SI of ≥50. Moreover, the remaining methoxylated derivatives were deemed moderately active (EC_{50} between 26.21 and 9.07 μM). Interestingly, despite the common 3,4-OMe substituents that resemble veraguensin (**1**, EC_{50} 2.67 μM), compound **4d** displayed only moderate efficacy against the trypomastigote parasite (EC_{50} 24.63 μM), being one of the less favourable modifications introduced in the tetrahydrofuran series, along with the unsubstituted counterpart **4a** (EC_{50} 37.31 μM). A similar effect was achieved for grandisin (**2**, EC_{50} 28.59 μM) when compared to the corresponding derivative **4b** bearing the 3,4,5-OMe group, with closer parasite inhibition (EC_{50} 26.21 μM).

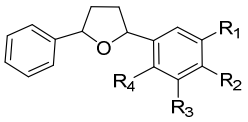
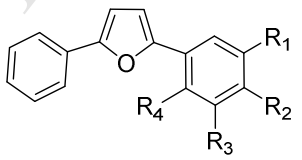
Based on the promising results obtained with derivatives **4e** and **4f** against Tulahuen strains, we also investigated their activities against the more infective *T. cruzi* Y strain and found compound **4e** preserves high anti-trypanosomal activity (EC_{50} 1.05 μM) while **4f** exhibited only EC_{50} 34.22 μM. Therefore, the maintenance of a single methoxylated phenyl ring in the set of compounds **4**, rather than two methoxylated phenyl rings, besides the absence of the 3,4-dimethyl tetrahydrofuran substituents as observed in natural products **1** and **2**, has proved to be an interesting simplification strategy applied to this class of neolignans.

The influence of the number and position of the methoxyl group on the aromatic ring was also evident in the 2,5-diaryl-furan series **5a-g**. Among them, the 3,5-OMe derivative **5c** showed the strongest anti-trypanosomal activity of the current study, with EC_{50} 0.01 μM, 75-fold better than the corresponding compound **4e** (2,4-OMe), the most potent in the tetrahydrofuran series (EC_{50} 0.75 μM), and more than a 150-fold better than benznidazole (EC_{50} 1.65 μM); Table 1. Besides the nanomolar activity, the cytotoxicity of **5c** (CC_{50}) was not detectable until 250 μM, resulting in a highly selective agent (SI>250) for further optimisation and development. In addition, the significant inhibitory effect on the parasite growth displayed by compounds **5d** (3,4-OMe) and **5e** (2,4-OMe), with EC_{50} values of 1.34 and 1.12 μM, respectively, also revealed the importance of the dimethoxylated substitution pattern on the

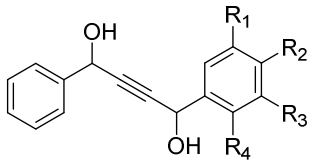
aromatic ring in the 2,5-diaryl-furan series. On the other hand, the trimethoxylated **5b** (29.10 μM) and unsubstituted counterparts **5a** (21 μM) of this 2,5-diaryl-furan series led to decrease in inhibitory activity, being less active than the monomethoxylated products **5f** (3.34 μM) and **5g** (4.75 μM), bearing monomethoxylated groups at the 3 and 4 positions, respectively. The tests carried out with the more active diarylfurans **5c-g** against *T. cruzi* Y strain confirm the better profile of compound **5c** (EC_{50} 4.07 μM) compared to **5d-g** (19.16-35.87 μM), despite the lower than nanomolar range found with Tulahuen strain (0.01 μM) and reference benznidazole (0.04 μM) against the same Y strain.

In order to investigate the impact of the tetrahydrofuran/furan central cores on anti-trypanosomal activities, we also evaluated the anti-trypanosomal and cytotoxic activities of the precursors related to rigid compounds (**8a-g**, **9a-g**) and flexible (**10a-g**) open chain intermediates, having different connection pattern between the substituted and unsubstituted aromatic rings that can adopt different spatial orientations (Table 1). It was found that among 1,4-diaryl-2-butyn-1,4-diol precursors (**8a-g**) bearing a rigid alkynyl link, derivatives having close methoxyl aryl substitution (3,4,5-OMe, **8b**; 3,5-OMe **8c** and 3,4-OMe, **8d**) to grandisin (2) and veraguensin (1), just in one ring, showed significant activities of 4.88, 4.98 and 8.98 μM, respectively, whereas others were moderately active (39.11-13.97 μM). This set of compounds was not cytotoxic in the tested concentrations up to 250 μM. Compared to the distinguished activities displayed by some rigid 1,4-diaryl-2-butyn-1,4-diol precursors (**8b-d**), the anti-trypanosomal activity induced by the corresponding reduced 1,4-diarylbutane-1,4-diol set (**10a-g**), containing a flexible central chain linking both aromatic rings, was significantly lower (19.88 to 193.10 μM). In addition to the decreased activities displayed by **10b** (3,4,5-OMe) and **10e** (2,4-OMe) derivatives, it was also accompanied by increased toxicity. Oxidation of these intermediates (**10a-g**) rendered the constrained 1,4-diarylbutane-1,4-diketone analogues **9a-g**, which highlights **9c** (3,5-OMe) and **9e** (2,4-OMe), with EC_{50} 1.69/15.91 and 7.79/1.76 μM for Tulahuen/Y strains, respectively, as the most active of this series, despite lower activities than those observed for the same methoxylated pattern required for higher activity in the furan (**5c**) and tetrahydrofuran (**4e**) series. Therefore, taking into account the highest potency exhibited by series **4**, **5**, **8** and **9**, compared to the 1,4-diarylbutane-1,4-diol **10**, it should be inferred that a preferential constrained central core is essential for anti-trypanosomal activity, since in the 1,4-diketones series a specific rigid spatial arrangement might be adopted owing to the conjugated dienol tautomer formation. Besides, this diketone series does not display cytotoxicity at concentrations lower than 250 μM.

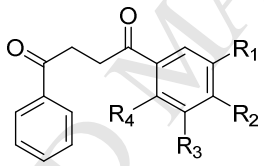
Table 1. Anti-trypanosomal activities of novel diaryl-tetrahydrofuran (**4a-g**) and -furan **(5a-g)** derivatives of natural products veraguensin (**1**) and grandisin (**2**) and the corresponding precursors, **8-10**(**a-g**), against *T. cruzi* Trypomastigote Tulahuen strain, and the most active compounds against Y strain.

Compound					Trypomastigote form of Tulahuen strain EC_{50} (µM)	Cytotoxicity CC_{50} (µM)	SI CC_{50}/ EC_{50})	Trypomastigote form of Y strain EC_{50}(µM)	Amastigote form of Tulahuen strain EC_{50}(µM)
	R_1	R_2	R_3	R_4					
4a	H	H	H	H	37.31±24.1	NC		-	
4b	OMe	OMe	OMe	H	26.21±7.3	NC	-	-	-
4c	OMe	H	OMe	H	12.09±1.9	NC	-	-	-
4d	OMe	OMe	H	H	24.63±0.3	NC	-	-	-
4e	H	OMe	H	OMe	0.75±0.6	NC	-	1.05± 0.05	19.77± 0.3
4f	H	H	OMe	H	2.29±1.6	NC	-	34.22± 0.1	125.7± 2.0
4g	H	OMe	H	H	9.07±3.7	NC	-	-	-
									
5a	H	H	H	H	21.00±2.4	193.60 ±62.3	9.2	-	-
5b	OMe	OMe	OMe	H	29.10±5.1	NC	-	-	-
5c	OMe	H	OMe	H	0.01±0.007	NC	-	4.08± 0.3	133.10± 4.0
5d	OMe	OMe	H	H	1.34±0.9	NC	-	32.63± 0.6	125.8± 3.2
5e	H	OMe	H	OMe	1.12±0.2	NC	-	35.87± 2.6	19.00± 2.7
5f	H	H	OMe	H	3.34±2.6	NC	-	34.22± 0.6	125.60± 0.2

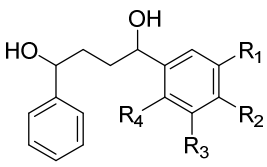
5g	H	OMe	H	H	4.75±1.4	NC	-	19.16± 0.6	127.00± 4.5



8a	H	H	H	H	18.67 ± 2.6	NC	-	-	-
8b	OMe	OMe	OMe	H	4.88 ± 1.5	NC	-	97.11± 5.3	-
8c	OMe	H	OMe	H	4.98 ±1.5	78.6 ±6.4	15.8	-	-
8d	OMe	OMe	H	H	8.98 ± 4.5	NC	-	-	-
8e	H	OMe	H	OMe	37.87 ± 0.4	NC	-	-	-
8f	H	H	OMe	H	39.11 ±6.4	NC	-	-	-
8g	H	OMe	H	H	13.97±2.7	NC	-	-	-



9a	H	H	H	H	198.60± 12.1	219.90 ±12.7	1.10	-	-
9b	OMe	OMe	OMe	H	36.87±3.3	NC	-	-	-
9c	OMe	H	OMe	H	1.69±0.05	NC	-	15.91± 3.9	-
9d	OMe	OMe	H	H	35.96±5.7	NC	-	-	-
9e	H	OMe	H	OMe	7.79±0.8	NC	-	1.76± 1.9	-
9f	H	H	OMe	H	18.31±0.02	NC	-	-	-
9g	H	OMe	H	H	15.87±7.2	NC	-	-	-



10a	H	H	H	H	33.16±4.31	165.90± 22.2	5.0	-	-
10b	OMe	OMe	OMe	H	77.50±7.9	115.20 ±38.20	1.5	-	-
10c	OMe	H	OMe	H	67.62±0.8	NC	-	-	-
10d	OMe	OMe	H	H	83.12±11.7	NC	-	-	-
10e	H	OMe	H	OMe	19.88±6.0	236.40± 51.9	11.9	-	-
10f	H	H	OMe	H	193.10±8.8	NC	-	-	-
10g	H	OMe	H	H	68.07±6.7	NC	-	-	-

NC: Not Cytotoxicity until 250 µM; EC_{50}: Effective Concentration of compounds causing 50% parasite death, being veraguensin (**1**): EC_{50} 2.67± 0.5 µM, grandisin (**2**): EC_{50} 28.59± 0.4 µM and benznidazole: EC_{50} 1.65± 0.3 µM, using trypomastigote *T. cruzi* Tulahuen strain. CC_{50}: Cytotoxic Concentration of compounds causing death to 50% of viable spleen cells (isolated from C57BL/6 mice). SI: Selective Index: relation between CC_{50} (µM) (Cytotoxic Concentration of compounds) and EC_{50} (µM) (Effective Concentration of compounds against Trypomastigote form of Tulahuen Strain).

To find out whether hit compounds (**4e**, **4f**, **5c-g**) might affect not only trypomastigote forms, but also the replicative *T. cruzi* amastigote form located inside the host cell, we performed additional assays using infected LLC-MK2 cells. As a result, the 2,4-dimethoxy-diaryltetrahydrofuran **4e** and 2,4-dimethoxy-diarylfuran **5e** were the most potent of the selected compounds when compared to the benznidazole drug (EC_{50} 9.78 µM), with similar EC_{50} values of 19.77 and 19 µM, respectively. These findings are in agreement with the activities established in the tetrahydrofuran series against trypomastigote parasite, in which compound **4e** was also the most active. Conversely, the most active furan derivative **5c** against *T. cruzi* trypomastigote displayed only weak activity against the amastigote (EC_{50} 133.1 µM), about 13-fold lower than benznidazole, while the most potent **5e**, identified in the amastigote assay, was the second most potent against *T. cruzi* trypomastigote tests (Figure 2).

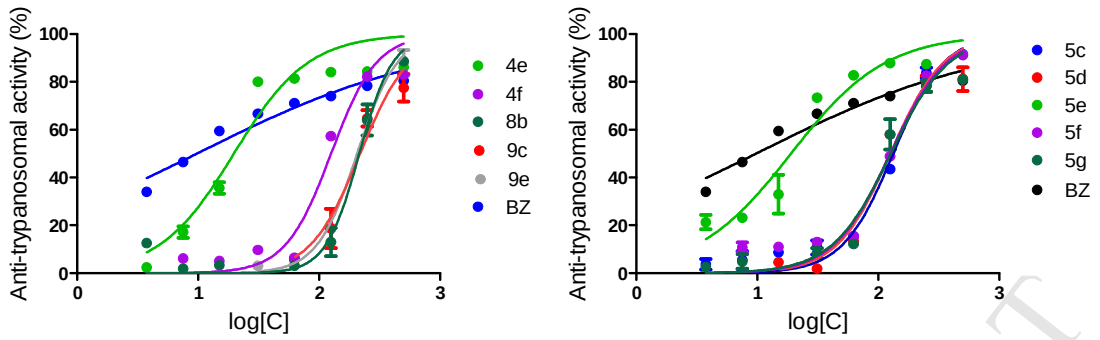


Figure 2. Biological evaluation of the most active compounds identified in the trypomastigote assays towards amastigote form of *T. cruzi,* Tulahuen strain.

2.3 2D-QSAR studies

Five molecular descriptors were selected by OPS-PLS methodology[70] and the model (**1**) was obtained using 29 compounds with no outliers. Based on three mutually orthogonal variables with 63.445% of information (LV1: 34.497%; LV2: 14.066%; LV3: 14.882%), obtained from these descriptors, partial least squares (PLS) was conveniently applied to the model [71]. The obtained and predicted values of each compound are reported in the Supplementary Material (Table S1).

$pEC_{50} = 16.937 - 6.916(SpMin3\_Bh(e)) - 0.878(VE1\_B(s)) + 61.221(JGI6) - 4.261(MATS3m) + 0.152(CATS2D\_05\_AL)$ **(1)**

$n = 29$; $R^2 = 0.679$; $RMSEC = 0.354$; $F = 17.627$; $Q^2_{LOO} = 0.507$; $RMSECV = 0.439$.

The internal validation shows that the model explains and predicts some information, and fulfills the requirements of literature ($R^2 > 0.6$; $Q^2_{LOO} > 0.5$), and shows, by the result of the F test ($F_{critical} = 2.991$, $p = 3$, and $n - p - 1 = 25$, $\alpha = 0.05$), that the model is reliable and significant. Moreover, the difference between R^2 and Q^2_{LOO} (0.172) [72] suggests the model does not undergo data over fitting. The model was also approved in the internal quality robustness tests (LNO cross-validation) and chance correlation y-randomisation (Figure 3) [72-74].

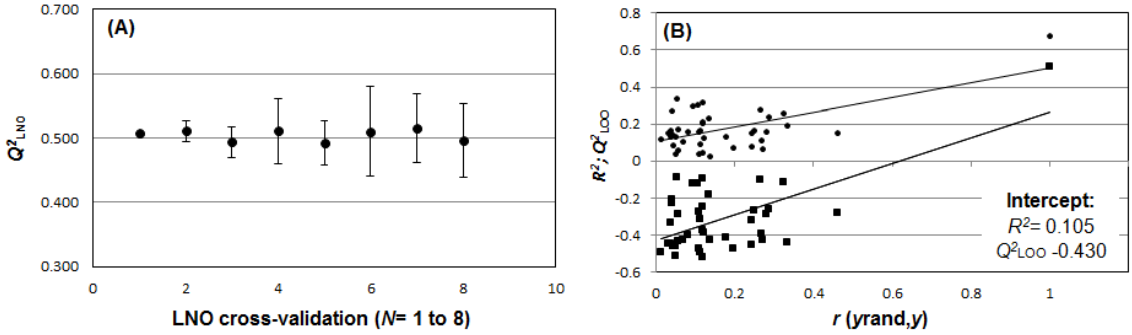


Figure 3. Plots of LNO cross-validation (A) and y-randomisation tests (B).

Regarding the external validation, it indicates that Model 1 is realistic and applicable for prediction purposes. Despite the lower correlation in the cross-validation process, the external validation of the model showed a good external predictive power, with R^2_{pred} = 0.848, *RMSEP* = 0.190, average r_m^2(LOO)-scaled = 0.753, Δr_m^2(LOO)-scaled = 0.062, $|R_0^2 - R'^2_0|$ = 0.004, k = 0.965 and k' = 1.036 [34,75]. Besides the better values of R^2_{pred} in comparison with Q^2_{LOO}, it is important to highlight that there is no relationship between internal and external predictability (i.e., a model with low internal predictability may present high external predictability, and *vice versa*); a paradox previously described by Kubinyi [76,77]. The results of Golbraikh and Tropsha statistics and the RmSquare metrics also confirm the good external predictability of the model [34,72].

All five selected descriptors are topological descriptors, which encode molecular information such as size, shape, symmetry, branching, and rigidity, besides atom type and bond multiplicity [78]. The order of importance of each descriptor is based on absolute values of their autoscaled coefficients, (|0.647| x SpMin3_Bh(e) > |0.398| x VE1_B(s) > |0.369| x JGI6 > |0.364| x CATS2D_05_AL > |0.264| x MATS3m). The meaning of each descriptor and their corresponding values are shown in Tables S2 and S3, respectively, in the Supplementary Material.

It is worth noting that the two most important descriptors, (SpMin3_Bh(e) and VE1_B(s)) are related to molecular electronegativity and intrinsic electropological state [79]. Both may influence the anti-trypanosomal activity according to the position of the methoxyl group on the aromatic ring, even in those with the same number of methoxyl groups, such as compounds **8d** (3,4-OMe) and **8e** (2,4-OMe), **10d** (3,4-OMe) and **10e** (2,4-OMe). Taking into account that SpMin3_Bh (e) is obtained based on Burden matrices (B(w)), where the diagonal elements are atomic carbon-scaled properties (w_i/w_C),[79] a large number of electronegative

atoms in the molecule may lead to a decrease of the descriptor value in the model since trimethoxylated derivatives showed lower activity than the remaining products, with the exception of **8b** (3,4,5-OMe). Alternatively, the electronic effect (σ) of the methoxyl group, with electron-donating effect at the *para*-position and electron-withdrawing at the *meta*-position[80] may contribute to the high and moderate activities of compounds **4f** (3-OMe), **5d** (3,4-OMe), **5f** (3-OMe) e **9c** (3,5-OMe), along with the most active of the series **5c** (3,5-OMe), containing two methoxyl groups at the *meta*-position, indicating that the decreased electron density on the ring is actually relevant for the biological activity, but to a certain extent, which can be evidenced by the negative sign of the descriptor SpMin3_Bh(e). Similarly, VE1_B(s) is also obtained from Burden matrix, with close interpretation, and from the sum of the number of vertices (i.e., atoms) present in an H-depleted molecular graph [79]. This indicates that the more structures are branched (i.e., the larger the number of methoxyl groups) the greater the tendency to be less active.

It was also evident that the influence of the number of methoxyl groups on the activity is not enough to explain the entire model. The position of the substituent also plays an important role due to the combination of electronic effects on the aromatic ring, as described by the third descriptor (JGI6), which encodes the total charge transfer between atoms placed at topological distance 6 (aromatic ring topology). Furthermore, the fourth descriptor (CATS2D_05_AL) comprises topological distances formed by five bonds (*lags*) between hydrogen bond acceptors and lipophilic chemical groups (i.e., two main characteristics of the methoxyl group). The positive signal of this descriptor in model 1 means the more this topological feature is present, the greater the tendency of the molecule to belong to the group of the most active derivatives. Amongst the most active products, it is important to highlight the prevalence of a methoxyl substitution pattern of R_1 and R_3, such as in compounds **9c** and **5c**, or R_2 and R_4, such as in **9e, 4e, 5e** and **10e**, which is in agreement with the biological results.

Finally, the descriptor (MATS3m) involving atomic mass can be considered the least important. An increase of the absolute value of this descriptor tends to increase the activity of derivatives, indicating the tendency of the higher molecular weight compounds to be slightly more active. In fact, unsubstituted and monosubstituted compounds (**9a**, **5a**, **10a**, **4a**, **8f**, **10g**, **10f**) represent 41% of the less active compounds ($pEC_{50} < 4.729$), while the most active (**4e**, **4f**, **5f**, **5g**, **9f**) make up 29% ($pEC_{50} > 4.729$).

2.4 Trypanothione reductase activity

Although the phenotypic screening of diaryltetrahydrofuran and diarylfuran against the trypomastigote and amastigote forms of *T. cruzi* (Tulahuen and Y strains) revealed potent inhibitors, the identification of the corresponding molecular target are still essential for subsequent lead optimization process and drug development [12].

Thus, the role played by furan [31] and nitrofuran derivatives, in particular, the redox-cycling drugs nitrofurazone and nifurtimox [81,82] and the subversive substrates related to guanidine-containing nitro-furans (under aerobic conditions) [83,84], on inhibition of *T. cruzi* Trypanothione reductase (TR) motivated us to investigate the effect of the most active anti-trypanosomal compounds from our series (IC_{50} < 12 µM) on this target, taking into account the unique metabolic pathway and anti-oxidant functions of TR that provides a cellular reducing environment to protect the parasite against oxidative stress [85].

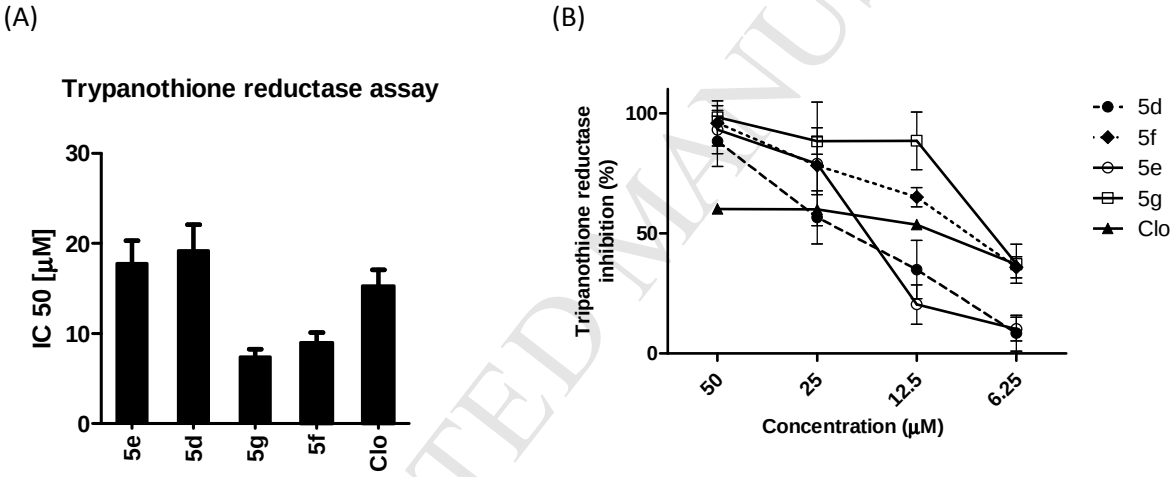


Figure 4. (A) Inhibition of trypanothione reductase by compounds **5d-g** and comparison of their IC_{50} values with clomipramine (Clo) (B) Corresponding curves of inhibition.

The assessment of TR activity was conducted using a continuous colorimetric microplate assay (time-dependent TR inhibition of 30 min) based on the capacity of the enzyme to reduce the trypanothione disulphide substrate (TS_2) to the di-thiol product $T(SH)_2$ in the presence of the NAPH cofactor. After incubation, the newly formed $T(SH)_2$ is re-oxidized back to the original substrate (TS_2) by the addition of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), which releases the chromophore 2-nitro-5-mercaptobenzoic acid (TNB), detected at 412 nm [86]. Thus, the assessment of the prospective diarylfurans and diaryl-tetrahydrofurans TR inhibitors (50,0 µM concentration) revealed the diarylfuran series as the most active

inhibitors, highlighting compounds **5d-e** (IC_{50} 19.2 and 17.7 μM) and **5f-g** (IC_{50} 8.9 and 7.4 μM), respectively, with similar or 2-fold higher than clomipramine (IC_{50} 15.2 μM) (Figure 4). It was evident that a monomethoxylated pattern of the diarylfuran core (at either *-para* or *-meta* position) had a significant impact on the TR inhibition.

Unexpectedly, compound **5c** that displayed the strongest activity against Tulahuen and Y strains of *T. cruzi* proved to be inactive, suggesting that its mechanism of action does not involve TR activity inhibition. Furthermore, similar results were found for the tetra-hydrofuran derivatives that showed no TR inhibition at tested concentrations and additional studies are necessary to elucidate their mechanism of action.

3. Conclusions

Based on natural neolignan products, a series of fourteen novel diaryl-tetrahydrofuran (**4a**-**g**) and -furan derivatives (**5a**-**g**) were synthesised as a simplified approach for the development of more potent anti-trypanosomal agents. The cyclisation/dehydration intramolecular S_N2 reaction between a hydroxyl group and a leaving group was pursued to produce functionalised cyclic ethers as a mixture of equal amounts of *cis*- and *trans*-2,5-diphenyl-tetrahydrofurans **4a-g**. In addition, microwave-assisted acceleration of the Pal-Knorr classical reaction allowed the preparation of 2,5-diphenylfurans **5a-g** in good to moderate yields.

Preliminary biological results suggested that derivatives with aryl-methoxy groups linked by a rigid central core, as observed in series **4**, **5**, **8** and **9**, may adopt a favourable orientation for target interactions in order to contribute with a beneficial entropic effect, which is not achieved by similar aryl methoxyl groups connected by a flexible link (series **10**). Based on the significant impact of the number and variable positions of the aryl methoxyl groups on the anti-trypanosomal activities, it should be inferred that the presence of a dimethoxy substituent pattern in all series had a stronger anti-trypanosomal effect than the corresponding monomethoxylated ones, which in turn, were better inhibitors than the trimethoxylated (with exception of compound **8b**) and unsubstituted counterparts.

2D-QSAR studies reinforce that the electronic effect of the methoxyl groups and their corresponding substitution pattern (number and position) on the aromatic ring have a significant influence on the overall electronic distribution in the final structure, affecting anti-trypanosomal activities. These findings open up the possibility of further chemical modifications to obtain more active derivatives, keeping, for instance, methoxyl group at R_1

and varying R_2 with similar molecular weight and electron donating groups, such as fluoro, cyano, trifluoromethyl and chlorine groups.

The identification of the most potent anti-trypanosomal non-chiral derivative in the diarylfuran series (**5c**) suggests that the configuration of the 2,5-tetrahydrofuran carbons in series **4** or even in the corresponding carbons of the rigid alkynyl open chain **8** are not crucial for parasite growth inhibition. Additionally, the simplified approach that maintained a single methoxylated aromatic ring rather than two, besides the elimination of two stereogenic centres (3,4-dimethyl groups of this class of neolignans natural products **1** and **2**) afforded more active derivatives against both Tulahuen and Y strains, along with strong effects on trypomastigote and amastigote forms of *T. cruzi*.

Finally, the assessment of trypanothione reductase inhibition using the most active anti-trypanosomal derivatives, highlight the potential of some diarylfurans to inhibit parasite growth by targeting its defense mechanism against oxidative stress through TR inhibition. In summary, this study demonstrates the relevance of simplified neolignan-based derivatives to act as stronger anti-trypanosomal agents than the parent natural product and, some of them, as trypanothione reductase inhibitors.

4. Experimental

4.1. General information

All chemicals were purchased as reagent grade and used without further purification and solvents were dried according to standard procedures [87]. Reactions were monitored by thin layer chromatography (TLC) on pre-coated silica gel aluminum plates (60 GF254, Merck) with the indicated eluents. Compounds were visualized under UV light (254 nm) and/or dipping in ethanol–sulfuric acid (95:5, v/v), followed by heating the plate for a few minutes. Column chromatography was performed on silica gel 60 (20–63 μm) or on a Biotage Horizon High-Performance FLASH Chromatography system using 12 mm or 25 mm flash cartridges with the indicated eluents. ^{1}H and ^{13}C Nuclear magnetic resonance spectra were recorded on Bruker Advance DRX 300 (300 MHz), DPX 400 (400 MHz) or DPX 500 (500 MHz) spectrometers and chemical shifts (δ) were expressed in parts per million (ppm), using tetramethylsilane (TMS) as internal standard. Accurate mass electrospray ionization mass spectra (ESI-HRMS) were obtained using positive or negative ionization modes on a Bruker Daltonics MicroOTOF II ESI-TOF mass spectrometer.

4.2 Synthesis

4.2.1 General procedure for synthesis of derivatives 2,5-diphenyl-tetrahydrofuran, 4a-g.

A solution of 1,4-diphenylbutane-1,4-diol derivatives (**10a-g**) in $CHCl_3$ was cooled at 0 ºC and trifluoracetic acid solution (5 eq) in $CHCl_3$ was added drop wise and stirred for 2 h. After that, the temperature was raised to ambient temperature for complete consumption of starting material. The reaction mixture was quenched into saturated aqueous NaOH 10% solution and extracted with dichloromethane. The organic layer was dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure and the mixture was purified by flash column chromatography (silica gel), hexane/ethyl acetate (9:1 v/v).

2,5-diphenyl-tetrahydrofuran (4a)[50-55]

Following procedure described in section 4.2.4, the reaction of 1,4-diphenylbutane-1,4-diol (**10a**) (20.3 mg, 0.085 mmol) in $CHCl_3$ (2.0 mL) with TFA (32 µL, 0.425 mmol) in $CHCl_3$ (1.0 mL) gave the product **4a** as a yellow oil, (8.06 mg, 0.036 mmol, 42%). ^{1}H NMR (400 MHz, $CDCl_3$): δ: 1.79-1.97 (4H, m, H-8, H-9); 4.68-4.79 (2H, m, H-7, H-10); 7.24-7.30 (2H, m, H-4); 7.30-7.39 (8H, m, H-2, H-3, H-5, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ: 35.2, 35.9 (C-8, C-9); 74.3, 74.7 (C-7, C-10); 125.8 (C-2, C-6); 127.5, (C-4); 128.5 (C-3, C-5); 144.5, 144.6 (C-1, C-1'). ESI-HRMS: calcd for $C_{16}H_{17}O$ $[M^+H]^+$ 225.1279; found 225.1242.

2-phenyl-5-(3,4,5-trimethoxyphenyl)tetrahydrofuran (4b)

Following procedure described in section 4.2.4, the reaction of 1-phenyl-4-(3,4,5-trimethoxyphenyl)butane-1,4-diol (**10b**) (25.2 mg, 0.076 mmol) in $CHCl_3$ (2.0 mL) with TFA (29.1 µL, 0.380 mmol) in $CHCl_3$ (1.0 mL) gave the product **4b** as a yellow oil, (12.25 mg, 0.039 mmol, 52%). ^{1}H NMR (400 MHz, $CDCl_3$): δ: 1.95-2.08 (2H, m, H-8a, H-9a); 2.40-2.54 (2H, m, H-8b, H-9b); 3.84 (6H, s, OCH_3); 3.88 (3H, s, OCH_3); 5.00-5.12 (1H, m, H-7 or H-10); 5.17-5.30 (1H, m, H-7 or H-10); 6.65 (2H, d, *J* 3.1 Hz, H-2', H-6'); 7.26-7.31 (1H, m, H-4); 7.34-7.48 (4H, m, H-2, H-3, H-5, H-6).). ^{13}C NMR (100 MHz, $CDCl_3$)): δ:35.5 (C-8,C-9), 56.0 (C11, C13), 60.8 (C12), 81.3 (C7-C10), 102.6 (C1´, C5´), 124.9 (C1-C5), 127.2 (C3), 127.4 (C2-C4), 137.2 (C3´), 139.3 (C6´), 143.6 (C6), 153,3 (C2´, C4´). ESI-HRMS: calcd for $C_{21}H_{25}NaO_4$ $[M^+Na]^+$ 337.1416; found 337.1412.

2-(3,5-dimethoxyphenyl)-5-phenyltetrahydrofuran (4c)

Following procedure described in section 4.2.4, the reaction of 1-(3,5-dimethoxyphenyl)-4-phenylbutane-1,4-diol (**10c**) (20.7 mg, 0.068 mmol) in $CHCl_3$ (2.0 mL) with TFA (26.5 µL, 0.342 mmol) in $CHCl_3$ (1.0 mL) gave the product **4c** as a yellow oil, (5.7 mg, 0.020 mmol, 30%). ^{1}H NMR (400 MHz, $CDCl_3$): δ: 1.85-1.97 (2H, m, H-8a, H-9a); 2.29-2.42 (2H, m, H-8b, H-9b); 3.71 (3H, s, OCH_3); 3.73 (3H, s, OCH_3); 4.91-5.02 (1H, m, H-7 or H-10); 5.11-5.22 (1H, m, H-7 or H-10); 6.30 (1H, q, *J* 2.3 Hz, H-4'); 6.51 (1H, d, *J* 2.3 Hz, H-2' or H5'); 6.53 (1H, d, *J* 2.3 Hz, H-2' or H5'); 7.21-7.40 (5H, m, H-2, H-3, H-4, H-5, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ: 34.0, 34.3, 35.4 (C-8, C-9); 55.3 (O*C*H_3); 81.1, 81.2, 81.3, 81.4 (C-7, C-10); 99.1 (C-4'); 103.3, 103.9 (C-2', C-6'); 125.6, 126.0 (C-2, C-6); 127.2, 127.3 (C-4); 128.3, 128.4 (C-3, C-5); 145.7 (C-1, C-1'); 160.8 (C-3', C-5'). ESI-HRMS: calcd for $C_{18}H_{21}O_3$ $[M^+H]^+$ 285.1491; found 285.1487.

2-(3,4-dimethoxyphenyl)-5-phenyltetrahydrofuran (4d)[56,57]

Following procedure described in Section 4.2.4, the reaction of 1-(3,4-dimethoxyphenyl)-4-phenylbutane-1,4-diol (**10d**) (30.8 mg, 0.102 mmol) in $CHCl_3$ (3.0 mL) with TFA (39.0 µL, 0.510 mmol) in $CHCl_3$ (1.0 mL) gave the product **4d** as a yellow oil, (18.75 mg, 0.066 mmol, 65%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 1.96-2.06 (2H, m, H-8a, H-9a); 2.40-2.51 (2H, m, H-8b, H-9b); 3.84 (3H, s, OCH_3); 3.88 (3H, s, OCH_3); 4.99-5.11 (1H, m, H-7 or H-10); 5.24 (1H, dt, *J* 6.8 Hz, *J* 7.1 Hz, H-7 or H-10); 6.65 (2H, d, *J* 3.1 Hz, H-2', H4'); 7.26-7.32 (1H, m, H-6'); 7.32-7.45 (5H, m, H-2, H-3, H-4, H-5, H-6). ^{13}C NMR (75 MHz, $CDCl_3$): δ: 34.2, 35.5, 35.6 (C-8, C-9); 55.8, 55.9 (O*C*H_3); 81.1, 81.2 (C-7, C-10); 109.0, 109.5, 111.0 (C-2', C-5'); 117.8, 118.1 (C-6'); 125.5, 125.9 (C-2, C-6); 127.1, 127.2 (C-4); 128.3 (C-3, C-5); 136.1, 135.6, 142.5, 143.7 (C-1, C-1'); 148.2, 149.0 (C-3', C-4'). ESI-HRMS: calcd for $C_{18}H_{21}O_3$ $[M^+H]^+$ 285.1491; found 285.1488.

2-(2,4-dimethoxyphenyl)-5-phenyltetrahydrofuran (4e)

Following procedure described in section 4.2.4, the reaction of 1-(2,4-dimethoxyphenyl)-4-phenylbutane-1,4-diol (**10e**) (9.8 mg, 0.032 mmol) in $CHCl_3$ (1.0 mL) with TFA (13.0 µL, 0.160 mmol) in $CHCl_3$ (1.0 mL) gave the product **4e** as a yellow oil, (3.98 mg, 0.014 mmol, 45%). ^{1}H NMR (400 MHz, $CDCl_3$): δ: 1.82-2.00 (2H, m, H-8a, H-9a); 2.34-2.53 (2H, m, H-8b, H-9b); 3.81 (3H, s, OCH_3); 3.82 (3H, s, OCH_3); 5.00 (0.5 H, t, *J* 7.0 Hz, H-7 or H-10); 5.26 (1H, t, *J* 6.7 Hz, H-7 or H-10); 5.46 (0.5 H, t, *J* 6.7 Hz, H-7 or H-10); 6.45-6.53 (2H, m, H-3', H-5'); 7.27-7.53 (6H, m, H-2, H-3, H-4, H-5, H-6, H-6'). ^{13}C NMR (100 MHz, $CDCl_3$): δ: 33.1, 33.9, 34.3, 35.5 (C-8, C-9), 55.3, 55.4 (O*C*H_3), 80.8, 80.9 (C-7, C-10), 98.3, 98.4 (C-3'), 103.8 (C-5'), 124.0, 124.6 (C-1'), 125.6 (C-2, C-6), 126.4, 126.7 (C-6'), 127.0, 127.2 (C-4), 128.3 (C-3, C-5), 143.2, 144.0 (C-1),

157.2, 157.3, 159.8, 159.9 (C-2', C-4'). ESI-HRMS: calcd for $C_{18}H_{21}O_3$ $[M^+H]^+$ 285.1491; found 285.1488.

2-(3-methoxyphenyl)-5-phenyltetrahydrofuran (4f)

Following procedure described in section 4.2.4, the reaction of 1-(3-methoxyphenyl)-4-phenylbutane-1,4-diol (**10f**) (36.9 mg, 0.135 mmol) in $CHCl_3$ (3.0 mL) with TFA (51.8 µL, 0.677 mmol) in $CHCl_3$ (1.0 mL) gave the product **4f** as a yellow oil, (13.7 mg, 0.054 mmol, 40%). ^{1}H NMR (400 MHz, $CDCl_3$): δ: 1.86-1.96 (2H, m, H-8a, H-9a); 2.33-2.42 (2H, m, H-8b, H-9b); 3.74 (3H, s, OCH_3); 4.98 (1H, *J* 3.0 Hz, *J*, 4.0 Hz, *J* 7.0 Hz, H-7 or H-10); 5.16-5.21 (1H, m, H-7 or H-10); 6.72-6.77 (1H, m, H-6'); 6.89-6.97 (2H, m, H-2', H-4'); 7.18-7.23 (2H, m, H-4, H-5'); 7.26-7.40 (4H, m, H-2, H-3, H-5, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ: 34.3, 34.4, 35.6 (C-8, C-9); 55.2, 55.3 (O*C*H_3); 81.1, 81.2, 81.3, 81.4 (C-7, C-10); 111.0, 111.6 (C-2'); 112.6, 112.7 (C-4'); 117.9, 118.3 (C-6'); 125.6, 126.0 (C-2, C-6); 127.2, 127.3 (C-4); 128.4 (C-3, C-5); 129.4 (C-5'); 142.9, 143.6, 144.8,145.5 (C-1, C-1'); 159.7 (C-3'). ESI-HRMS: calcd for $C_{17}H_{18}NaO_2$ $[M^+Na]^+$ 277.1204; found 277.1236.

2-(4-methoxyphenyl)-5-phenyltetrahydrofuran (4g)[51,58-60]

Following procedure described in Section 4.2.4, the reaction of 1-(4-methoxyphenyl)-4-phenylbutane-1,4-diol (**10g**) (42.2 mg, 0.155 mmol) in $CHCl_3$ (3.0 mL) with TFA (59.3 µL, 0.775 mmol) in $CHCl_3$ (1.0 mL) gave the product **4g** as a yellow oil, (26.43 mg, 0.104 mmol, 67%). ^{1}H NMR (400 MHz, $CDCl_3$): δ: 1.84-1.95 (2H, m, H-8a, H-9a); 2.25-2.42 (2H, m, H-8b, H-9b); 3.72 (3H, s, OCH_3); 4.89-4.97 (1H, m, H-7 or H-10); 5.10-5.19 (1H, m, H-7 or H-10); 6.80-6.84 (2H, m, H-3', H-5'); 7.15-7.21 (1H, m, H-4); 7.25-7.37 (6H, m, H-2, H-3, H-5, H-6, H-2', H-6'). ^{13}C NMR (100 MHz, $CDCl_3$): δ: 34.3, 34.6, 35.6, 35.7 (C-8, C-9); 55.3 (O*C*H_3); 81.1, 81.2 (C-7, C-10); 113.8 (C-3', C-5'); 125.6, 126.0 (C-2, C-6); 127.0, 127.2 (C-4); 127.3, 127.4 (C-2', C-6'); 128.4 (C-3, C-5); 134.9, 135.6 (C-1'); 143.1, 143.8 (C-1); 158.9, 159.0 (C-4'). ESI-HRMS: calcd for $C_{17}H_{18}NaO_2$ $[M^+Na]^+$ 277.1204; found 277.1218.

4.2.2 General procedure for synthesis of derivatives 2,5-diphenylfuran, 5a-g.

A solution of 1,4-diphenylbutane-1,4-dione derivatives (**9a-g**) in MeCN was treated with triflic acid (1.1 equiv). The reaction mixture was stirred for 20 min at 80 ºC (sealed microwave tube) under microwave irradiation (100 W). After that, the reaction was quenched into saturated $NaHCO_3$ solution. The organic and aqueous phases were separated and the aqueous phase extracted with EtOAc (3 x 2.0 mL). The organic layer was dried over anhydrous $MgSO_4$.

Removal of the solvent under reduced pressure followed by purification by flash column chromatography (silica gel), hexane/ethyl acetate (9:1 v/v) afforded products (**5a-g**)

2,5-diphenylfuran (5a)[64,65]

Following procedure described in section 4.2.5, the reaction of 1,4-diphenylbutane-1,4-dione (**9a**) (31.5 mg, 0.132 mmol) in MeCN (2.0 mL) with TfOH (12.8 µL, 0.145 mmol) gave the product **5a** as a white solid, (21.35 mg, 0.097 mmol, 74%). ^{1}H NMR (400 MHz, $CDCl_3$): δ: 6.74 (2H, s, H-8, H-9); 7.27 (2H, ddt, *J* 1.3 Hz, *J* 1.9 Hz, *J* 7.4 Hz, H-4); 7.41 (4H, ddt, *J* 1.3 Hz, *J* 1.9 Hz, *J* 7.4 Hz, H-3, H-5); 7.75 (4H, ddt, *J* 1.2 Hz, *J* 1.9 Hz, *J* 7.3 Hz, H-2, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ: 107.2 (C-8, C-9); 123.7 (C-2, C-6); 127.3 (C-4); 128.7 (C-3, C-5); 130.8 (C-1); 153.4 (C-7, C-10). ESI-HRMS: calcd for $C_{16}H_{12}KO$ $[M^+K]^+$ 259.0525; found 259.0727.

2-phenyl-5-(3,4,5-trimethoxyphenyl)furan (5b)[66]

Following procedure described in section 4.2.5, the reaction of 1-phenyl-4-(3,4,5-trimethoxyphenyl)butane-1,4-dione (**9b**) (15.0 mg, 0.046 mmol) in MeCN (1.0 mL) with TfOH (5.0 µL, 0.051 mmol) gave the product **5b** as a yellow solid, (4.30 mg, 0.014 mmol, 31%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 3.89 (3H, s, OCH_3); 3.96 (6H, s, OCH_3); 6.68 (1H, d, *J* 3.5 Hz, H-8 or H-9); 6.74 (1H, d, *J* 3.5 Hz, H-8 or H-9); 6.96 (2H, s, H-2', H-6'); 7.30 (1H, m, H-4), 7.42 (2H, t_{app}, *J* 7.5 Hz, H-3, H-5), 7.74 (2H, m, H-2, H-6). ^{13}C NMR (75 MHz, $CDCl_3$): δ:56.2 (C11, C13), 61.0 (C12), 101.1 (C8, C9), 107.3, 107.0 (C1´, C5´), 123.7 (C1, C5), 126.5 (C3), 127.4 (C6), 128.7 (C2, C4), 130.7 (C6´),137.7 (C3´),153.2 (C8, C9), 153.6 (C2´, C4´). ESI-HRMS: calcd for $C_{19}H_{19}O_4$ $[M^+H]^+$ 311.1283; found 311.1265.

2-(3,5-dimethoxyphenyl)-5-phenylfuran (5c) [65]

Following procedure described in section 4.2.5, the reaction of 1-(3,5-dimethoxyphenyl)-4-phenylbutane-1,4-dione (**9c**) (27.2 mg, 0.091 mmol) in MeCN (1.5 mL) with TfOH (8.9 µL, 0.100 mmol) gave the product **5c** as a yellow solid, (13.16 mg, 0.047 mmol, 52%). ^{1}H NMR (400 MHz, $CDCl_3$): δ: 3.87 (6H, s, OCH_3); 6.41 (1H, t, *J* 2.3 Hz, H-4'); 6.73 (1H, d, *J* 3.5 Hz, H-8 or H-9); 6.74 (1H, d, *J* 3.5 Hz, H-8 or H-9); 6.91 (2H, d, *J* 2.3 Hz, H-2', H-6'); 7.28 (1H, dt, *J* 1.6 Hz, *J* 7.4 Hz, H-4); 7.41 (2H, t, *J* 7.4 Hz, H-3, H-5); 7.74 (2H, dd, *J* 1.6 Hz, *J* 7.4 Hz, H-2, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ: 55.4 (OCH_3); 99.6 (C-4'); 102.0 (C-2', C-6'); 107.2, 107.8 (C-8, C-9); 123.8 (C-2, C-6); 127.4 (C-4); 128.7 (C-3, C-5); 130.7 (C-1); 132.5 (C-1'); 153.1, 153.4 (C-7, C-10); 161.1 (C-3', C-5'). ESI-HRMS: calcd for $C_{18}H_{16}NaO_3$ $[M^+Na]^+$ 303.0997; found 303.0993.

2-(3,4-dimethoxyphenyl)-5-phenylfuran (5d)

Following procedure described in section 4.2.5, the reaction of 1-(3,4-dimethoxyphenyl)-4-phenylbutane-1,4-dione (**9d**) (21.8 mg, 0.073 mmol) in MeCN (1.5 mL) with TfOH (7.1 µL, 0.080 mmol) gave the product **5d** as a yellow solid, (15.41 mg, 0.055 mmol, 75%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 3.85 (3H, s, OCH_3); 3.93 (3H, s, OCH_3); 6.54 (1H, d, *J* 2.4 Hz, H-2'); 6.60 (1H, dd, *J* 8.6 Hz, *J* 2.4 Hz, H-6'); 6.74 (1H, d, *J* 3.4 Hz, H-8 or H-9); 6.88 (1H, d, *J* 3.4 Hz, H-8 or H-9); 7.24 (1H, ddt, *J* 1.2 Hz, *J* 1.7 Hz, *J* 7.4 Hz, H-4); 7.39 (2H, t_{app}, *J* 7.4 Hz, H-3, H-5), 7.74 (2H, m, H-2, H-6); 7.89 (1H, d, *J* 8.6 Hz, H-5'). ^{13}C NMR (75 MHz, $CDCl_3$): δ: 56.0 (OCH_3); 106.0, 107.3 (C-8, C-9); 111.5 (C-2'); 116.7 (C-5'); 123.6 (C-2, C-6, C-6'); 124.1 (C-1'); 127.2 (C-4); 128.7 (C-3, C-5); 130.9 (C-1); 148.8, 149.2 (C-3', C-4'); 152.9, 153.4 (C-7, C-10). ESI-HRMS: calcd for $C_{18}H_{16}NaO_3$ $[M^+Na]^+$ 303.0997; found 303.0999.

2-(2,4-dimethoxyphenyl)-5-phenylfuran (5e)

Following procedure described in section 4.2.5, the reaction of 1-(2,4-dimethoxyphenyl)-4-phenylbutane-1,4-dione (**9e**) (22.0 mg, 0.074 mmol) in MeCN (1.5 mL) with TfOH (7.2 µL, 0.081 mmol) gave the product **5e** as a yellow solid, (14.40 mg, 0.050 mmol, 68%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 3.92 (3H, s, OCH_3); 3.98 (3H, s, OCH_3); 6.62 (1H, d, *J* 3.5 Hz, H-8 or H-9); 6.72 (1H, d, *J* 3.5 Hz, H-8 or H-9); 6.91 (1H, d, *J* 8.4 Hz, H-5'), 7.22-7.29 (2H, m, H-4, H-3'), 7.32 (1H, dd, *J* 8.4 Hz, *J* 2.0 Hz, H-6'), 7.40 (2H, t_{app}, *J* 7.5 Hz, H-3, H-5); 7.73 (2H, dd, *J* 7.5 Hz, J 1.3 Hz, H-2, H-6). ^{13}C NMR (75 MHz, $CDCl_3$): δ: 55.4 (OCH_3); 98.7 (C-3'); 104.8, 107.3 (C-8, C-9); 110.2 (C-5'); 113.3 (C-1'); 123.6 (C-2, C-6); 126.6 (C-6'); 126.9 (C-4); 128.6 (C-3, C-5); 131.0 (C-1); 150.0; 151.6 (C-7; C-10); 156.7, 160.0 (C-2', C-4'). ESI-HRMS: calcd for $C_{18}H_{16}NaO_3$ $[M^+Na]^+$ 303.0997; found 303.0994.

2-(3-methoxyphenyl)-5-phenylfuran (5f)

Following procedure described in section 4.2.5, the reaction of 1-(3-methoxyphenyl)-4-phenylbutane-1,4-dione (**9f**) (26.4 mg, 0.098 mmol) in MeCN (1.5 mL) with TfOH (7.2 µL, 0.081 mmol) gave the product **5f** as a yellow solid, (15.75 mg, 0.063 mmol, 64%). ^{1}H NMR (400 MHz, $CDCl_3$): δ: 3.88 (3H, s, OCH_3); 6.74 (2H, s, H-8, H-9); 6.83 (1H, dt, *J* 2.5 Hz, *J* 8.4 Hz, H-4'); 7.24-7.34 (4H, m, H-4, H-2', H-5', H-6'); 7.40 (2H, t_{app}, *J* 7.3 Hz, H-3, H-5); 7.75 (2H, dt, *J* 1.3 Hz, *J* 7.3 Hz, H-2, H-6). ^{13}C NMR (100 MHz, $CDCl_3$): δ: 55.3 (OCH_3); 107.2, 107.6 (C-8, C-9); 109.2 (C-2'); 112.9 (C-4'); 116.4 (C-6'); 123.7 (C-2, C-6); 127.4 (C-4); 128.7 (C-3, C-5); 129.8 (C-5'); 130.7,

132.0 (C-1, C-1'); 153.1, 153.4 (C-7, C-10); 159.9 (C-3'). ESI-HRMS: calcd for $C_{17}H_{15}O_2$ $[M^+H]^+$ 251.1072; found 251.1066.

2-(4-methoxyphenyl)-5-phenylfuran (5g) [66]

Following procedure described in section 4.2.5, the reaction of 1-(4-methoxyphenyl)-4-phenylbutane-1,4-dione (**9g**) (24.3 mg, 0.091 mmol) in MeCN (1.5 mL) with TfOH (8.8 µL, 0.100 mmol) gave the product **5g** as a yellow solid, (12.0 mg, 0.048 mmol, 53%). ^{1}H NMR (300 MHz, $CDCl_3$): δ: 3.84 (3H, s, OCH_3); 6.60 (1H, d, *J* 3.5 Hz, H-8 or H-9); 6.71 (1H, d, *J* 3.5 Hz, H-8 or H-9); 6.94 (2H, d, *J* 8.9 Hz, H-3', H-5'); 7.25 (1H, ddt, *J* 1.2 Hz, *J* 1.9 Hz,*J* 7.5 Hz, H-4); 7.39 (2H, t_{app}, *J* 7.5 Hz, H-3, H-5); 7.68 (2H, dt, *J* 1.5 Hz, *J* 8.9 Hz, H-2', H-6'); 7.73 (2H, m, H-2, H-6). ^{13}C NMR (75 MHz, $CDCl_3$): δ: 55.3 (OCH_3); 105.6, 107.2 (C-8, C-9); 114.2 (C-3', C-5'); 123.5 (C-2, C-6); 123.9 (C-1'); 125.2 (C-2', C-6'); 127.1 (C-4); 128.7 (C-3, C-5); 130.9 (C-1); 152.7, 153.4 (C-7, C-10); 159.0 (C-4'). ESI-HRMS: calcd for $C_{17}H_{15}O_2$ $[M^+H]^+$ 251.1072; found 251.1056.

4.3 Biological evaluation

Trypomastigote assay

To evaluate the anti-trypanosomal activity, cell-derived trypomastigote forms of *T. cruzi*, Tulahuen strain stably expressing the β-galactosidase gene from Escherichia coli (Tulahuen-lacZ), 5×10^5 trypomastigotes, were cultured in 96-well plates and incubated for 4 days at 37°C with the synthesized compounds (products **4-5a-g** and intermediates **8-10a-g**), natural products veraguensin (**1**) and grandisin (**2**) and benznidazole drug (Sigma-Aldrich) at serial concentrations in a range between 500 to 3.75 µM. Thereafter, 50 µL of PBS containing 0.5% of Triton X-100 and 100 µM chlorophenol Red-β-D-galactoside (CPRG-Sigma) were added. Plates were incubated at 37°C for 4 h and absorbance was read at 570 nm.[67] Results of parasite viability were measured based on the catalysis of CPRG by β-galactosidase and the experiments were performed in duplicates. To test the compounds against *T. cruzi* Y strain, trypomastigotes were platted in 96 well flat bottom plate at concentration of 1×10^6 parasites mL^{-1} and the more active products on trypomastigote parasite, **4e-f** and **5c-g** and intermediates **8b**, **9c**, were platted in serial concentrations (500 to 3.75 µM). After 24 h, the parasites were removed and counted in Neubauer chamber as previously described.[68,69]

Amastigote assay

Anti-trypanosomal activities against amastigote form of *T. cruzi* Tulahuen Lac-Z strain were performed *in vitro*. LLC-MK2 cells were resuspended in RPMI 1640 without phenol red (Gibco)

supplemented with 5% FBS and antibiotics. LLC-MK2 cells were harvested at 2 x 10^4 cell $\times mL^{-1}$ in 96 wells plate for 24 h and infected in a parasite/cell ratio 3:1 with the trypomastigote of *T. cruzi* Tulahuen strain at 37°C. After 4 hours post-infection, cells were hard washed to remove the extracellular parasites. The most active compounds against the trypomastigote forms (products **4e-f**, **5c-g** and intermediates **8b** and **9c**), and veraguensin (**1**) and benznidazole drug (Sigma-Aldrich) were added at concentrations of 500 µM to 0.24 µM, twenty-four hours after infection and cultivated at 37°C. After 4 days of culture, the cells were extensive washed and added 50µL of PBS containing 0.5% of Triton X-100 and 100µM chlorophenol red-β-D-galactosidase (CPRG, Sigma Aldrich). Plates were incubated at 37°C for 4 h and absorbance was measured at 570 nm. Results of parasite viability were determined at the base of the catalysis of CPRG by β-galactosidase. Amastigotes without treatment was considered negative control and infected cells treated with benznidazole, positive control.

Cytotoxicity assay

The cytotoxicity of compounds (products **4-5a-g** and intermediates **8-10a-g**), natural products veraguensin (**1**) and grandisin (**2**) and benznidazole drug (Sigma-Aldrich) was evaluated on spleen cells isolated from C57BL/6 mice at a concentration range of 250 to 1.50 µM. Spleens were macerated in RPMI 1640 medium (Gibco-BRL Life Technologies, Grand Island, NY) and incubated for 5 min with red blood cell lysis buffer (one part of 0.17M Tris–HCl and nine parts of 0.16 M ammonium chloride). The isolated cells were centrifuged at 1500 rpm for 10 min and resuspended in RPMI medium containing 5% fetal bovine serum (Life Technologies Inc., Bethesda, MD) and antibiotics (Sigma Chemical Co., St. Louis). The spleen cells were seeded flat-bottom 96-well plates at 6.5×10^6/mL cells/well with different concentrations of the synthesized compounds at 37°C for 24 h [88,89]. Tween 20 at 1% was used as cell death positive control and benznidazole drug (Roche) was used as a reference drug. After 24 h, the cells were incubated with 10 µg/mL propidium iodide (Sigma) and acquired using a FACSCantoII (Becton-Dickinson Immunocytometry System Inc., San Jose, CA, USA). Data analysis was performed using FlowJo software (Ashland, Oregon, USA). The experiments were also performed in triplicates.

Trypanothione reductase assay

Recombinant trypanothione reductase from *T. cruzi* (TcTR) was expressed in *Escherichia coli* BL21DE3 and purified by affinity chromatography. TR assays were performed as described by Hamilton[86] in 96-well flat bottom micro plates (final volume = 240 µL), containing TcTR (5 m-unit), HEPES (40 mM pH 7.5), NADPH (Sigma-Aldrich) (0.15 mM) and

EDTA (1 mM), TS_2 (Sigma-Aldrich) (1 µM) and tested compounds (50 µM), diluted in DMSO. The reaction mixture was pre-incubated at 27ºC for 30 min and after 10µL of DNTB was added, absorbance was read at 412 nm for 30 min in 5 min interval at 27ºC in a TECAN® Infinite M200 micro plate reader. Clomipramine and DMSO 1% were used as positive and negative controls, respectively. Compounds that inhibited > 50% of TR activity were tested again in serial dilution (50µM -1.5µM) and IC_{50} values were calculated by a non-linear regression using the GraphPad Prism program. All assays were done in triplicate and repeated three times [86].

4.4 Quantitative structure-activity relationships studies

Because most of the compounds are racemic mixtures, a set of more simple molecular descriptors (as constitutional, topological and molecular descriptors) were obtained using only the Simplified Molecular Input Line Entry System (SMILES) in the Dragon 6. The absolute stereochemistry of the derivatives was not considered for generation of the strings. Next, it was carried out a variable reduction step, to eliminate descriptors with irrelevant or redundant information. Thus, the following features found in some descriptors were excluded: (i) constant values; (ii) near-constant values; (iii) standard deviation of less than 0.001; (iv) at least one missing value; (v) a pair correlation larger than or equal to 0.90; and (vi) absolute Pearson's correlation coefficient ($|r|$) values with the biological activity vector ***y*** (in the –log EC_{50}, or pEC_{50}, format) less than 0.15. The first steps were also carried out in Dragon 6, and the last step in 2D-QSAR Modeling [90]. A final verification was carried out by visual inspection, using a resultant matrix with 104 descriptors. Variable selection was performed using the ordered predictor selection (OPS) algorithm.[49] The models were constructed based on the partial least squares (PLS). Statistical tools to validate the quality of the fit, significance, and predicting power of the models were applied in order to evaluate the capacity of the models to generate reliable predictions of the biological activity under study to find out new active derivatives [91]. The validation approach comprised the internal and external steps, using 29 and 5 compounds, respectively. The set compounds selected for external validation was chosen according to their biological activities encompassing a range of 2.423 logaritimic units. The external validation results were obtained from the Xternal Validation Metric Calculator program [92]. In order to maintain the quality of the model, compound **5c** was removed from the study for presenting a pEC_{50} value outside the range of the biological activity variation, higher than 1.875 logaritimic units in relation to the second more active derivative **4e**.

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Supplementary data

Supplementary data (synthesis of precursors **8**-**10** (**a**-**g**), 1H, 13C NMR, DEPT-135, HMQC, HMBC and HRMS (ES+) spectra for all compounds) associated with this article can be found, in the online version, at ... These data include MOL files of the most important compounds described in this article.

Rerefences

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Highlights

Two series of diaryl-tetrahydrofuran and -furan derivatives were synthesized to impair *Trypanosoma cruzi* growth and survive.

The anti-trypanosomal activity and cytotoxicity of these compounds were evaluated.

Compounds **4e** and **5c** showed strong activities against *T. cruzi* Tulahuen and Y strains and high selective index.

2D-QSAR studies revealed the effect of the rigid central core and dimethoxy-aryl substituents on the anti-trypanosomal activity.

Some diaryl-furan derivatives proved to be potent inhibitors of the parasite Trypanothione reductase.